



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US98/14603 <b>(22) International Filing Date:</b> 17 July 1998 (17.07.98) <b>(30) Priority Data:</b> 08/897,236                      17 July 1997 (17.07.97)                      US <b>(71) Applicants:</b> REGENERON PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707 (US). THE REGENT OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3350 (US). <b>(72) Inventors:</b> STAHL, Neil; RD #10, Kent Shore Drive, Carmel, NY 10512 (US). HARLAND, Richard, M.; 402 Birchwood Drive, Moraga, CA 94556 (US). ECONOMIDES, Aris, N.; 12 Mt. Morris Park West, New York, NY 10027 (US). <b>(74) Agents:</b> COBERT, Robert, J.; Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US) et al.		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> MODIFIED DORSAL TISSUE AFFECTING FACTOR AND COMPOSITIONS <b>(57) Abstract</b> <p>Described are modified Noggin muteins, compositions comprising the muteins, and DNA or RNA sequences comprising coding (sense) or antisense sequences for the muteins.</p>		

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Claims

1. Human Noggin (SEQ ID NO. 2) modified by a deletion of amino acid residues 138-144.
2. An isolated nucleic acid molecule encoding the modified human Noggin of claim 1.
3. An isolated nucleic acid molecule of claim 2, which is a recombinant DNA molecule operatively linked to an expression control sequence.
4. A host cell transformed with the recombinant DNA molecule of claim 3.
5. A method for producing a modified Noggin molecule comprising: (a) growing a recombinant host cell containing the DNA molecule of claim 3, so that the DNA molecule is expressed by the host cell to produce the modified Noggin molecule and (b) isolating the expressed, modified Noggin molecule.
6. The method according to claim 5, wherein said host cell is a eukaryotic cell.
7. The method according to claim 5, wherein said host cell is a prokaryotic cell.

8. A composition comprising human Noggin according to claim 1, and a carrier.
9. A method of treatment comprising administering to a patient an effective amount of the composition of claim 8.
10. Human Noggin (SEQ ID NO. 2) modified by a deletion of amino acid residues 133-144.
11. An isolated nucleic acid molecule encoding the modified human Noggin of claim 10.
12. An isolated nucleic acid molecule of claim 11, which is a recombinant DNA molecule operatively linked to an expression control sequence.
13. A host cell transformed with the recombinant DNA molecule of claim 12.
14. A method for producing a modified Noggin molecule comprising: (a) growing a recombinant host cell containing the DNA molecule of claim 12, so that the DNA molecule is expressed by the host cell to produce the modified Noggin molecule and (b) isolating the expressed, modified Noggin molecule.

15. The method according to claim 14, wherein said host cell is a eukaryotic cell.
16. The method according to claim 14, wherein said host cell is a prokaryotic cell.
17. A composition comprising human Noggin according to claim 2 and a carrier.
18. A method of treatment comprising administering to a patient an effective amount of the composition of claim 17.
19. Biologically active noggin polypeptide modified by the deletion of the amino acid sequence KKLRRK.
20. An isolated nucleic acid molecule encoding the noggin polypeptide of claim 19.
21. An isolated nucleic acid molecule of claim 20, which is a recombinant DNA molecule operatively linked to an expression control sequence.
22. A host cell transformed with the recombinant DNA molecule of claim 21.
23. A method for producing a modified Noggin molecule comprising: (a) growing a recombinant host cell containing

the DNA molecule of claim 22, so that the DNA molecule is expressed by the host cell to produce the modified Noggin molecule and (b) isolating the expressed, modified Noggin molecule.

24. The method according to claim 23, wherein said host cell is a eukaryotic cell.
25. The method according to claim 23, wherein said host cell is a prokaryotic cell.
26. A composition comprising biologically active Noggin according to claim 19 and a carrier.
27. A method of treatment comprising administering to a patient an effective amount of the composition of claim 26.
28. Modified Noggin of claim 1, 10 or 19 conjugated to polyethylene glycol (PEG).
29. A composition comprising modified Noggin according to claim 28 and a carrier.
30. A method of treatment comprising administering to a patient an effective amount of the composition of claim 29.
31. The method of claim 9, 18, 27 or 30 for treating

Fibrodysplasia Ossificans Progressiva (FOP).

32. The method of claim 9, 18, 27 or 30 for treating a disease or disorder involving abnormal bone growth.
33. The method of claim 32 wherein the abnormal bone growth is the pathological growth of bone following hip replacement surgery, trauma, burns or spinal cord injury.
34. Use of a modified Noggin as defined in claim 1, 10, 19 or 28 in the manufacture of a medicament for the treatment of a disease or disorder affecting bone.
35. A modified Noggin according to claim 1, 10, 19 or 28, a nucleic acid molecule according to claim 2, 11 or 20, or a composition according to claim 8, 17, 26, or 29, for use in a method of treatment of the human body or in a method of diagnosis.

### MODIFIED DORSAL TISSUE AFFECTING FACTOR AND COMPOSITIONS

This International Application claims priority of U.S. Serial No. 897,236 filed July 17, 1997, the disclosure of which is hereby  
5 incorporated by reference. Throughout this application, various publications are referenced. The disclosures of those publications, in their entireties, are hereby incorporated by reference into the subject application.

10 This invention generally relates to a growth factor with dorsal growth inducing activity, and more particularly to a modified form of the Spemann organizer signal Noggin, to compositions comprising the modified Noggin, and to DNA or RNA sequences comprising coding (sense) or antisense sequences for the modified  
15 Noggin.

### BACKGROUND OF THE INVENTION

Growth factors are substances, such as polypeptide hormones,  
20 which affect the growth of defined populations of animal cells in vivo or in vitro, but which are not nutrient substances. Proteins involved in the growth or differentiation of tissues may promote or inhibit growth or differentiation, and thus the general term "growth  
25 factor" includes cytokines and trophic factors.

Growth factors, their receptors, DNA or RNA coding or antisense sequences therefore, and fragments thereof, are useful in a number



of therapeutic, clinical, research, diagnostic, and drug design applications. See, for example, U.S. Patent 4,857,637, issued August 15, 1989 (method for immunizing an animal against its growth hormone receptor); U.S. Patent 4,933,294, issued June 12, 1990 (assays and therapies involving the human EGF receptor); U.S. Patent 5,030,576, issued July 9, 1991 (the role of receptors and receptor hybrids in drug design and drug screening by the pharmaceutical industry); U.S. Patent 5,087,616, issued February 11, 1992 (method for destroying tumor cells using a composition comprising a growth factor conjugate); U.S. Patent 5,098,833, issued March 24, 1992 (expression systems useful in therapeutic or diagnostic compositions); and International Application Publication No. W092/05254, published April 2, 1992 (various aspects of isolation, preparation, and applications for a novel neurotrophic factor); each of which is incorporated herein by reference.

The Spemann organizer induces neural tissue from dorsal ectoderm and dorsalizes lateral and ventral mesoderm in Xenopus. The first molecule to have the properties expected of a Spemann organizer signal was identified in an expression screen for activities that induce dorsal structures in Xenopus embryos and was called Noggin (Smith, W.C. and Harland, R.M. Cell 70: 829-840 (1992)). Organizer signals such as Noggin may be antagonized by members of the bone morphogenetic protein (BMP) class of the transforming growth factor beta (TGF- $\beta$ ) gene superfamily. It was recently reported that Noggin protein binds BMP-4 with high affinity and can abolish BMP-4 activity by blocking binding to cognate cell surface

receptors (Zimmerman, L.B., et al., Cell 86: 599-606 (1996)).

In addition to their roles in normal bone formation, the BMPs appear to be involved in diseases in which they promote abnormal bone growth. For example, BMPs have been reported to play a causative role in the disease known as Fibrodysplasia Ossificans Progressiva (FOP), in which patients grow an abnormal "second skeleton" that prevents any movement.

10

#### DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B - Nucleotide sequence (SEQ ID NO:1) for the human noggin gene and deduced amino acid sequence (SEQ ID NO: 2).

15 The KKLRRK deletion that is described in Example 9 (hNG $\Delta$ 138-144) as adequate for reducing the interaction with heparin to that expected for ionic effects is encoded beginning at nucleotide 415 (AAG ...).

20 Figure 2A - Experimental design: competent animal cap (AC) ectoderm was dissected from staged embryos as shown. St10.5 dorsal and ventral AC and ventral marginal zones (VMZ) also dissected as shown. Explants were washed once in low Ca/Mg Ringers (LCMR) solution and then placed in treatment medium containing factor diluted in LCMR + 0.5%BSA. Explants cultured to  
25 late stages (St20+) were removed from treatment medium 6-16 hours after the start of treatment and placed in LCMR. When

explants reached the desired stage they were either harvested for RNA, or they were fixed for whole mount *in situ* hybridization or antibody staining.

5 Figure 2B - Neural induction by noggin in the absence of muscle.  
Lanes 1-3 show specific fragments protected by *N*-CAM,  $\beta$ -tubulin, and XIF-3 probes respectively in whole St24 embryo RNA. Lanes 4-8 show protection by the mixture of these three probes while lanes 9-13 show protection by an actin probe on tRNA(t), St24 embryo  
10 RNA (E), and RNA collected from St9 AC treated with 50pM activin (A), 25% of 20 fold concentrated control CHO cell medium (C) or 25% of 20 fold concentrated noggin conditioned CHO cell medium (N). Ubiquitously expressed cytoskeletal actin used as a loading control shows that RNA levels in all treatments are comparable  
15 (lanes 11-13).

Figure 3 - SDS-PAGE (12%) run under reducing conditions. Proteins were visualized by silver staining. Lane 1 shows molecular size standards. Lanes 2-7 show 0.0, 0.1, 0.2, 0.5, 1.0 and 2.0  $\mu$ g of  
20 purified human noggin.

Figure 4A - Time course of animal caps treated with purified noggin vs. activin; direct vs indirect neural induction. Animal caps were dissected as shown in Figure 2A and treated with LCMR + 0.5%  
25 BSA (U), a 20% dilution of activin conditioned COS cell medium (A), or 1 $\mu$ g/ml purified human noggin (N). RNA isolated from treated animal caps (lanes 2-13) along with St22 whole embryo RNA

(lane1) and tRNA (lane 14) was probed for *N-CAM*,  $\beta$ -tubulin, muscle and cytoskeletal actins, collagen type II, and EF-1a.

5 Figure 4B - Expression of early mesoderm markers in activin but not noggin induced animal caps. Animal caps were dissected from St8 embryos, treated as described in (Figure 4A), and harvested at St11. Lanes 1 and 2 respectively show *goosecoid* and *Xbra* probe protection by St10.5 whole embryo RNA. Lanes 3-6 show protection by a mix of these two probes. Relative RNA levels are demonstrated  
10 by separate *EF-1 $\alpha$*  probe protection.

Figure 4C - Plasmid directed gastrula stage noggin expression directly induces neural tissue. One cell stage embryos were injected with 20pg of pCSKA*lacZ* or pCSKA*noggin* into the animal  
15 pole. Animal caps from injected embryos were dissected at St8-9 and cultured until St20, when they were harvested for analysis by RNase protection.

Figure 5 - Responsiveness of dorsal and ventral animal caps to  
20 neural induction by noggin. St 10.5 ventral and dorsal animal caps were dissected as shown in Figures 2A-2B. Dorsal and ventral animal caps were treated with activin medium (DA,VA) or 1 $\mu$ g/ml human noggin (DN, VN) and harvested at St26 for RNase protection analysis using *N-CAM*,  $\beta$ -tubulin, and actin as probes.

25 Figure 6 - Dose response of ventral marginal zones and animal caps to human noggin protein. St 10.5 VMZs and St9 animal caps

were dissected as shown in Figure 2A, and treated with 0, 1, 10, 50, 200, and 1000 ug/ml of human noggin (lanes 3-8 and 10-15 respectively). RNA from treated explants and control whole embryos aged to St26 was then analyzed by RNase protection, using the probes *N-CAM*,  $\beta$ -tubulin, actin and collagen type II. In this experiment, muscle induction at the dose of 1ng/ml is stronger than at 10ng/ml, and there is a low level of muscle actin expression in the uninduced VMZs. This could be due to experimental variability since in repeated experiments we saw muscle induction only at the doses of 50ng/ml and above.

Figures 7A to 7L - *In situ* hybridization and antibody staining. Tailbud embryos stained for *NCAM* showing side and dorsal views (7A, 7B); *NCAM* RNA is only detected in the neural tube, and not the somites. For comparison, somites of a tailbud embryo stain for muscle actin, dorsal view (7C). Neural specific 6F11 antibody staining at St30 (7D-7F). Some cement gland pigment remained in these embryos after bleaching as seen in (7D), however this pigment is distinct from antibody staining. The inner mass of staining in the noggin treated animal caps is due to the 6F11 antibody detection. Cement gland specific *XAG-1* transcripts detected at St23 (7G-7i), and anterior brain *otxA* transcripts detected at St35 (7J-7L) in whole embryos at (7D,7G,7J), human noggin treated (1 $\mu$ g/ml) animal caps (7E,7H,7K), and untreated animal caps (7F,7i,7L).

Figure 8 - Reverse phase HPLC profile of two refolded isoforms of

noggin. The refolded noggin solution was applied onto a Brownlee Aquapore AX-300, 0.46 x 22 cmHPLC column at a flow rate of 1 ml/min. The column was equilibrated with solvent A containing 0.1% TFA in water. Solvent B was 0.1% TRA in acetonitrile. The column was developed according to the following protocol: a) 2 min isocratically at 95% of solvent A-5% of solvent B; 60 min linear gradient to 65% of solvent B and 35% of solvent A. Correctly refolded noggin elutes earlier at 44%-46% solvent B.

10 Figure 9 - Reverse-phase HPLC chromatography characterization of recombinant noggin refolded and purified from *E. coli*. Conditions as in the legend to Figure 8.

Figure 10 - Recombinant noggin produced in *E. coli* and in insect cells analyzed by 12.5% SDS-PAGE. Lanes H, L: High and low molecular weight markers of the indicated size, respectively. Lanes 1,2: Recombinant noggin produced in *E. coli* and in insect cells respectively, treated with 2-mercaptoethanol before electrophoresis. The slower mobility of noggin from insect cells corresponds to the size increase that would occur due to N-linked glycosylation at the single consensus site. Lanes 2,3: Recombinant noggin produced in *E. coli* and in insect cells respectively, not treated with 2-mercaptoethanol before electrophoresis.

25 Figure 11 - Circular dichroism spectra of recombinant noggin produced in *E. coli* (--), and in insect cells (-).

Figure 12 - Ventral marginal zone assay showing induction of muscle actin mRNA after exposure to human noggin (0.01, 0.05, 0.2  $\mu$ g/ml) produced in baculovirus, a mock transfected culture of baculovirus (0.02, 1  $\mu$ g/ml) or human noggin produced in *E.coli* (0.1, 0.5, 2, or 10  $\mu$ g/ml).

Figures 13A and 13B - Nucleotide sequence (SEQ ID NO:10) for the mouse noggin gene and deduced amino acid sequence (SEQ ID NO:11).

FIGURE 14 - Amino acid sequence of hNG $\Delta$ 133-144Fc

The putative signal peptide sequence of human noggin (hNG) is shown in italics.

(†) marks the position of cysteines (C), except as noted below.

(¥) marks the position of N-linked glycosylation sites.

( $\Delta$ ) marks the position in which the  $\Delta$ 133-144 deletion was created. The sequence of amino acids 133 to 144 in the wild-type hNG is KKQRLSKKLRRK and may be referred to as the 'basic region' of hNG.

The CYS involved in inter-chain disulfide bridges in hNG is marked in bold (...SECKCSC...).

The position of the Ser-Gly bridge that connects the hNG $\Delta$ 133-144 sequence with the constant region of human IgG1 (Fc) is shown in bold (SG). The sequence of the Fc domain is shown underlined.

( $\diamond$ ) marks the two cysteines (amino acids number 371 and 374) of the IgG hinge preceding the Fc that form the inter-chain disulfide bridges that link two Fc domains of human IgG1.

(•) shows the position of the STOP codon.

**FIGURE 15 - hNG $\Delta$ 133-144Fc binds to human BMP4**

The activity of either hNG or hNG $\Delta$ 133-144Fc was tested by comparing their ability to bind to human Bone Morphogenetic Protein 4 (hBMP4).

hBMP4 (2  $\mu$ g/ml) was coated on ELISA plates (CORNING) by passive binding. Unbound hBMP4 was removed by washing four times with PBS, and the plates were blocked with 1% BSA in PBS. A standard curve of hNG-Fc was performed to show dose-dependent binding of hNG-Fc to hBMP4 and compared to identical amounts of hNG $\Delta$ 133-144Fc. After a 1 hour incubation unbound hNG-Fc or hNG $\Delta$ 133-144Fc was removed by washing four times with PBS, and 0.5  $\mu$ g/ml anti-human IgG•Alkaline Phosphatase conjugate (anti-Fc•AP) was added to each well. After a 1 hour incubation unbound anti-Fc•AP was removed by washing four times with TBS+0.1% Tween and then Alkaline Phosphatase substrate (para-nitrophenyl phosphate; Sigma) was added. Alkaline Phosphatase converts this substrate to a product whose production can be monitored by measuring Absorbance at 405 nm. The ability of hNG or hNG $\Delta$ 133-144Fc to bind to BMP4 was visualized by comparing A405 units.

Note: There is no binding of hNG-Fc or hNG $\Delta$ 133-144Fc to the plates if hBMP4 is omitted.

**FIGURE 16 - hNG $\Delta$ 133-144Fc binds to heparin with reduced affinity**

Approximately 1  $\mu$ g of hNG-Fc, hNG $\Delta$ 138-144Fc, or hNG $\Delta$ 133-144Fc each were incubated with 50  $\mu$ l heparin•agarose (Pierce) in 1 ml



1% BSA in PBS. After 1 hour of incubation at room temperature the heparin•agarose beads were precipitated by centrifugation, resuspended in PBS and moved to new tubes. Subsequently each pellet was sequentially washed with 100  $\mu$ l of 0.1, 0.25, 0.5, 0.75, and 1.0 M NaCl and the supernatant derived from each of these steps was kept for loading on a 4 to 12% NuPAGE/MES gels (Novex) under reducing conditions. 1/5 of each supernatant and the resuspended heparin•agarose pellet was loaded onto the gels. The Fc-tagged hNGs were visualized by western blotting using a Horse Radish Peroxidase conjugated anti-human IgG antibody (Rockland, Inc.) followed by chemiluminescent detection (Pierce).

FIGURE 17A - hNG $\Delta$ 133-144Fc pharmacokinetics in mice

0.1 mg of hNG $\Delta$ 133-144Fc were injected into six week old Balb/c male mice (2 mice, 0.1 mg/mouse) intra-peritoneously (ip). Prior to injection a sample of blood was collected from each mouse ('pre-bleed'). Subsequently, blood was drawn from the mice at 1, 3, 6 and 24 hours post-injection. Serum was prepared from these samples by following standard serological procedures, and the levels of biologically active hNG $\Delta$ 133-144Fc available in the sera of Balb/c mice after the ip-injection were determined by using a functional ELISA.

hBMP4 (2  $\mu$ g/ml) was coated on ELISA plates (Immulon4 from Dynatech) by passive binding. Unbound hBMP4 was removed by washing four times with PBS, and the plates were blocked with 1% BSA in PBS. A standard curve of hNG $\Delta$ 133-144Fc was performed.

After a 1 hour incubation unbound hNG-Fc or hNG $\Delta$ 133-144Fc was removed by washing four times with PBS, and 0.5  $\mu$ g/ml anti-human IgG•Alkaline Phosphatase conjugate (anti-Fc•AP) was added to the plate and processed as described above (Figure 16). The level of hNG $\Delta$ 133-144Fc in the sera collected at each time point was determined by performing serial dilutions of each serum sample on the hBMP4-coated ELISA plates, and detecting Fc-immunoreactivity using the assay described above. A405 units were then converted to concentrations of hNG $\Delta$ 133-144Fc and plotted as a function of time.

Note: The amount of serum proteins present in these assays did not interfere with the binding and detection of hNG $\Delta$ 133-144Fc.

#### FIGURE 17B - hNG $\Delta$ 133-144Fc pharmacokinetics in rats

1.0 mg of hNG $\Delta$ 133-144Fc was injected into a 250 gram adult male rat intravenously (iv). Prior to injection, a sample of blood was collected ('pre-bleed'). Subsequently, blood was drawn from the rat at 10, 30, 60, 120, and 240 minutes post-injection. Serum was prepared from these samples by following standard serological procedures and the levels of biologically active hNG $\Delta$ 133-144Fc available in the serum samples were determined by using the functional ELISA described in Figure 17A.

FIGURES 18A - 18C - Pharmacokinetic profiles of human noggin (hNG) and modified human noggin molecules.

### SUMMARY OF THE INVENTION

Polypeptides of the invention induce dorsal growth in vertebrates  
5 and can be prepared in soluble, physiologically active form for a  
number of therapeutic, clinical and diagnostic applications.

In a preferred embodiment, human noggin protein as set forth in  
Figures 1A-1B (SEQ ID NO: 2) or modified noggin protein (SEQ ID NO.  
10 23) as set forth, for example, in Figure 14, is prepared for use in  
therapeutic, clinical and diagnostic applications.

In another aspect of the present invention an oligonucleotide, such  
as cDNA, is provided having substantial similarity to SEQ ID NO:1.  
15 This oligonucleotide can be single or double stranded, be formed of  
DNA or RNA bases, and can be in the antisense direction with  
respect to SEQ ID NO. 1. SEQ ID NO:1 codes for a functional  
polypeptide that we have designated "noggin," which is capable of  
inducing dorsal development in vertebrates when expressed.

20 Noggin or fragments or muteins thereof (which also may be  
synthesized by in vitro methods) may be fused (by recombinant  
expression or in vitro covalent methods) to an immunogenic  
polypeptide and this, in turn, may be used to immunize an animal in  
25 order to raise antibodies against a noggin epitope. Anti-noggin is  
recoverable from the serum of immunized animals. Alternatively,  
monoclonal antibodies may be prepared from cells to the

immunized animal in conventional fashion. Antibodies identified by routine screening will bind to noggin but will not substantially cross-react with "wnt" or other growth factors. Immobilized anti-noggin antibodies are useful particularly in the diagnosis (in vitro or in vivo) or purification of noggin.

Substitutional, deletional, or insertional mutants of noggin may be prepared by in vitro or recombinant methods and screened for immuno-crossreactivity with noggin and for noggin antagonist or agonist activity.

Noggin or fragments or muteins thereof also may be derivatized in vitro in order to prepare immobilized noggin and labelled noggin, particularly for purposes of diagnosis of noggin or its antibodies, or for affinity purification of noggin antibodies.

The present invention further provides for expression of biologically active noggin molecules in prokaryotic and eukaryotic expression systems.

The present invention further provides for the production of noggin or fragments or muteins thereof in quantities sufficient for therapeutic and diagnostic applications. Likewise, anti-noggin antibodies may be utilized in therapeutic and diagnostic applications. For most purposes, it is preferable to use noggin genes or gene products from the same species for therapeutic or diagnostic purposes, although cross-species utility of noggin may

be useful in specific embodiments of the invention.

In additional embodiments, the noggin nucleic acids, proteins, and peptides of the invention may be used to induce neural tissue formation or block BMP activity in mammals.

#### DETAILED DESCRIPTION OF THE INVENTION

10 Throughout this application, when nucleic acid sequences that encode polypeptides are set forth, it is understood that the complementary strand of the coding sequence is thereby taught as well.

15 We have discovered a structurally unique growth factor that is readily available in substantially pure, soluble form. We have named the inventive polypeptide "noggin." This growth factor induces dorsal development and blocks BMP activity in vertebrates.

20 An earlier described family of proteins that also induces dorsal development are the "wnt" proteins. These, however, in contrast to noggin remain tenaciously bound to cell surfaces. Our initial work with noggin has been in *Xenopus* embryos; however, noggin is highly conserved among vertebrates, as our work with mouse noggin has demonstrated. The prior known FGF growth factor family is also  
25 known to be involved in early embryonic induction, but both the FGF proteins and their receptors are distinctly different from

noggin. Noggin modifies the actions of FGF (and also activin), for example by potentiating growth, and is thus particularly suggested in therapeutic compositions for use in combination with other growth factors (as therapeutic adjuvants), such as to modify or  
5 potentiate their effects.

We have cloned cDNA for noggin. The noggin cDNA contains a single reading frame encoding a 26 kDa protein with a hydrophobic amino-terminal sequence. Noggin is secreted. Noggin's cDNA encodes the  
10 protein as a 26 kDa protein, but we have determined that noggin is secreted in vivo, apparently as a dimeric glycoprotein with a starting apparent molecular weight of about 33 kDa (as the wild-type subunit). When not glycosylated, the monomeric unit has an apparent molecular weight on SDS PAGE of about 25-30 kDa.

15 We have cloned the gene for human noggin (Figures 1A-1B; SEQ ID NO: 1). The sequence codes for a protein which has noggin activity (SEQ ID NO: 2). The carboxy terminal region of noggin shows homology to a Kunitz-type protease inhibitor, indicating that  
20 noggin protein, or fragments thereof, may exhibit activities of a protease inhibitor.

We have been able to express biologically active noggin in both eukaryotic and prokaryotic host cells. Two expression systems we  
25 have successfully used to express biologically active noggin have been mammalian cell lines (COS and mouse 293). A third expression system is injection of synthetic mRNA into *Xenopus*

oocytes. In addition, we have successfully expressed biologically active human noggin in a prokaryotic system, E. coli, and in baculovirus.

5 Expression in these several different systems also illustrates the high degree of conservation for noggin. We have found, for example, substantial sequence similarity between frog noggin and mouse noggin with a number of completely conserved stretches. Thus, the following amino acid sequences represent completely conserved  
10 portions as between frog noggin and mouse noggin:

QMWLWSQTFCPVLY (SEQ ID NO:3);

RFWPRYVKVGSC (SEQ ID NO:4);

SKRSCSVPEGMVCK (SEQ ID NO:5);

LRWRCQRR (SEQ ID NO:6); and,

15 ISECKCSC (SEQ ID NO:7).

There is about 87% overall conservation between the mouse and frog sequences, and we have also observed a unique cysteine distribution between the two.

20 Noggin nucleic acids, or oligonucleotides, encode a noggin polypeptide or hybridize to such DNA and remain stably bound to it under stringent conditions and are greater than about 10 bases in length; provided, however, that such hybridizing nucleic acid is novel and unobvious over any prior art nucleic acid including that  
25 which encodes or is complementary to nucleic acid encoding other growth factors.

By "stringent conditions" is meant those which (1) employ low ionic strength and high temperature for washing, for example, 0.15 M NaCl/0.015 M sodium citrate/0.1% NaDodSo<sub>4</sub> at 50°C, or (2) use during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C.

By "substantial similarity," when we are referring to a nucleotide sequence, is meant cross hybridization of sequences under conditions of moderate stringency using a probe greater than 100 nucleotides long at 30°C in a standard buffer (Wahl et al., PNAS 76: 3683) and washes at 37°C in 300 mM NaCl, 30 mM sodium citrate, 0.2% SDS at pH 7. Alternatively, one is able to isolate, by polymerase chain reaction, a fragment of DNA coding for noggin or noggin family members when using primers of degenerate sequence that encode those SEQ ID NOS:3-7.

By "substantial similarity" when reference is made to proteins is that noggin from different species, or noggin family members within a species, will preserve the positions of cysteine residues in at least 80% of positions throughout the protein. Like the neurotrophin family, the sequence of the mature form of noggin and noggin related polypeptides will be identical in at least 40% of positions. Substantial similarity at the protein level includes an ability of a subject protein to compete with noggin for binding to



receptors and some (but not all) monoclonal antibodies raised against noggin epitopes.

The cloned cDNA for full sequence of mouse noggin as shown in  
5 Figures 13A-13B (SEQ ID NO: 10). The human sequence is  
designated herein as SEQ ID NO: 1. We have used RNA transcripts  
from the frog noggin clone to rescue embryos and return them to  
substantially normal development when the noggin RNA is injected  
10 into ventralized embryos. In high doses this results in excessive  
head development and it is for this reason we named the protein  
"noggin." In Northern blot analysis, the noggin cDNA hybridizes to  
two mRNAs that are expressed both maternally and zygotically.

When using nucleotide sequences coding for part or all of noggin in  
15 accordance with this invention, the length of the sequence should  
be at least sufficient in size to be capable of hybridizing with  
endogenous mRNA for the vertebrate's own noggin. Typically,  
sufficient sequence size (for example, for use as diagnostic  
probes) will be about 15 consecutive bases (DNA or RNA). In some  
20 diagnostic and therapeutic applications, one may wish to use  
nucleotide noggin coding sequences (analogous to all or a portion of  
SEQ ID NO: 10 or SEQ ID NO:1 or Figure 14 (SEQ ID NO. 23)) in the  
anti-sense direction with respect to either SEQ ID NOS: 10 or 1 or  
Figure 14 (SEQ ID NO. 23).

25 We suggest as a few preferred primers for amplifying noggin from  
other species (e.g. human):

5' Primer 1 SEQ ID NO: 12

C A A/G A C N T T C/T T G C/T C C N G T N

5' Primer 2 SEQ ID NO: 13

5 T T C/T T G G C C N C/A G N T A C/T G T N A A A/G G T N G G

5' Primer 3 SEQ ID NO: 14

C C N G A A/G G G N A T G G T N T G

3' Primer 1 SEQ ID NO: 15

C A N C/G T/A A/G C A C/T T T A/G C A C/T T C

10

3' Primer 2 SEQ ID NO: 16

C A N A C C A T N C C C/T T C N G G

3' Primer 3 SEQ ID NO: 17

C G/T N C G/T T/C T G G/A C A N C G/T C C A

15

where N represents a mixture of all four nucleotides and mixtures of two nucleotides are represented by alternates (e.g. A/G).

Although noggin transcript is not localized in the oocyte and  
20 cleavage stage embryo, zygotic transcripts are initially restricted to the presumptive dorsal mesoderm, and reach their highest levels at the gastrula stage in the dorsal lip of the blastopore (Spemann's organizer). In the neurula, noggin is transcribed in the notochord and prechordal mesoderm.

25

Without being bound by theory, we have formulated hypotheses about the embryological effects of noggin based on where it is

expressed, and on the effects of RNA injection in embryos. Since  
noggin is expressed in the Spemann organizer, we believe noggin to  
be a mediator of the effects of the Spemann organizer, namely  
neural induction and dorsalization of the mesoderm. We have  
5 shown that noggin is able to directly induce neural tissue  
formation. Since noggin is expressed in the notochord and head  
mesoderm, we believe noggin to influence either the dorsal-ventral  
pattern or anterior-posterior pattern of the neural plate. Since  
noggin is expressed in the branchial arch neural crest, we believe  
10 it may therefore influence whether neural crest cells deposit  
cartilage and also to influence later branchial arch growth and  
remodelling. Noggin is expressed in the tail fin neural crest, and  
since neural crest is required for growth of the fin, noggin may act  
as a growth factor for epidermis or mesenchyme.

15

Although much of our experimental work has involved rescue of  
embryonic development, because expression in the notochord  
persists in the growing tail bud and a discontinuous line of stained  
cells (indicating expression of noggin initiated at new sites) runs  
20 the length of the roof plate of the neural tube (and is also apparent  
in the head mesoderm), we believe noggin is expressed as an adult  
cell function also.

25

A number of applications for noggin are suggested from its  
properties.

The noggin cDNA should be useful as a diagnostic tool (such as use  
of oligonucleotides as primers in a PCR test to amplify those with

sequence similarities to the oligonucleotide primer, and to see how much noggin is present, e.g. primers such as 5' Primers 1-3 and 3' Primers 1-3).

- 5 Because noggin has a pattern of expression that suggests it is used to regulate cartilage production in the embryonic head, clinical uses to regulate cartilage and bone growth are suggested for noggin in therapeutic compositions and particularly in combination with other growth factors due to a property of noggin to potentiate  
10 at least some growth factors. Since neural crest cells are required for the tadpole fin to grow, noggin seems to be a growth factor for the tissue matrix and epidermis and should prove useful, for example, in wound healing compositions.
- 15 When one views noggin as ligand in complexes, then complexes in accordance with the invention include antibody bound to noggin, antibody bound to peptides derived from noggin, noggin bound to its receptor, or peptides derived from noggin bound to its receptor. Mutant forms of noggin, which are either more potent agonists or  
20 antagonists, or have improved properties such as increased bioavailability, are believed to be clinically useful. Such complexes of noggin and its binding protein partners will find uses in a number of applications.
- 25 Practice of this invention includes use of an oligonucleotide construct comprising a sequence coding for noggin and for a promoter sequence operatively linked to noggin in a mammalian,

bacterial or a viral expression vector. Expression and cloning vectors contain a nucleotide sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate  
5 independently of the host chromosomes, and includes origins of replication or autonomously replicating sequences. The well-known plasmid pBR322 is suitable for most gram negative bacteria, the 2 $\mu$  plasmid origin for yeast and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in  
10 mammalian cells.

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. Typically, this is a gene that encodes a protein necessary for the survival or growth of a host  
15 cell transformed with the vector. The presence of this gene ensures that any host cell which deletes the vector will not obtain an advantage in growth or reproduction over transformed hosts. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin,  
20 methotrexate or tetracycline, (b) complement auxotrophic deficiencies.

Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR) or thymidine kinase. Such markers  
25 enable the identification of cells which were competent to take up the noggin nucleic acid. The mammalian cell transformants are placed under selection pressure in which only the transformants

are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed.

- 5 Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of noggin can therefore be synthesized from the amplified DNA.

10

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium which contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell in this case is the

- 15 Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Nat. Acad. Sci., 77, 4216 (1980). The transformed cells then are exposed to increased levels of Mtx. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple
- 20 copies of other DNA comprising the expression vectors, such as the DNA encoding noggin. Alternatively, host cells transformed by an expression vector comprising DNA sequences encoding noggin and aminoglycoside 3' phosphotransferase (APH) protein can be selected by cell growth in medium containing an aminoglycosidic
- 25 antibiotic such as kanamycin or neomycin or G418. Because eukarotic cells do not normally express an endogenous APH activity, genes encoding APH protein, commonly referred to as neo

resistant genes, may be used as dominant selectable markers in a wide range of eukaryotic host cells, by which cells transformed by the vector can readily be identified.

5 Expression vectors, unlike cloning vectors, should contain a promoter which is recognized by the host organism and is operably linked to the noggin nucleic acid. Promoters are untranslated sequences located upstream from the start codon of a structural gene (generally within about 100 to 1000 bp) that control the  
10 transcription and translation of nucleic acid under their control. They typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a  
15 nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters can be operably linked to noggin encoding DNA by removing them from their gene of origin via restriction enzyme digestion, followed by insertion 5' to the start codon for  
20 noggin.

Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for  
25 a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription

of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, 5 contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

10 Transcription of noggin-encoding DNA in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma, cytomegalovirus, adenovirus, retroviruses, hepatitis-B virus, and most preferably Simian Virus 40 (SV40), or from heterologous mammalian promoters, e.g. the actin promoter. Of 15 course, promoters from the host cell or related species also are useful herein.

In particular embodiments of the invention expression of noggin in E. coli is preferably performed using vectors which comprise the 20 following: a lac UV5 promoter which may be controlled by the lactose operon repressor; a strong ribosome binding site, for example, the ribosome binding site of bacteriophage T7; a mutation in the replication control region of the plasmid which may increase copy number; and a mutation which limits the expression of the 25 antibiotic resistance protein.

In a preferred embodiment, noggin is expressed in a high copy



number kanamycin resistant pBR322-derived plasmid under the control of a lac UV5 promoter. In an additional preferred embodiment, noggin is expressed in baculovirus under the control of the polyhedrin promoter of Autographa californica Multiple  
5 Nuclear Polyhedrosis virus in insect host cells.

An object of the present invention is to provide novel modified Noggin molecules for the treatment of diseases or disorders including, but not limited to, Fibrodysplasia Ossificans Progressiva  
10 (FOP), as well as for treating abnormal bone growth, such as the pathological growth of bone following hip replacement surgery, trauma, burns or spinal cord injury.

A further object of the present invention is to provide a method for  
15 producing modified Noggin molecules, other than those specifically described herein, that have improved therapeutic properties.

These and other objects are achieved in accordance with the invention, whereby amino acid deletions in human Noggin protein  
20 enhance its therapeutic properties.

The present invention provides for Human Noggin modified by a deletion of amino acid residues 138-144 as well as Human Noggin modified by a deletion of amino acid residues 133-144. The  
25 invention also provides for an isolated nucleic acid molecule encoding a modified human Noggin. The isolated nucleic acid molecule may be a recombinant DNA molecule operatively linked to

an expression control sequence. The present invention also provides for a host cell transformed with the recombinant DNA molecule.

5 In addition, the subject invention provides for a method for producing a modified Noggin molecule comprising: (a) growing a recombinant host cell containing the DNA molecule so that the DNA molecule is expressed by the host cell to produce the modified  
10 Noggin molecule and (b) isolating the expressed, modified Noggin molecule. The method may be carried out in a eukaryotic cell or a prokaryotic cell.

The invention provides for a composition comprising a modified human Noggin and carrier as well as a method of treatment  
15 comprising administering to a patient an effective amount of the composition. Diseases or disorders that may be treated include, but are not limited to, Fibrodysplasia Ossificans Progressiva (FOP), and abnormal bone growth, such as the pathological growth of bone following hip replacement surgery, trauma, burns or spinal cord  
20 injury. Because noggin and modified noggin are known to bind and antagonize BMPs, the molecules of the invention may be used to regulate the processes involved in bone production and bone turnover.

25 Thus, according to the invention, certain amino acid deletions in the human Noggin protein result in a modified human Noggin protein that exhibits improved bioavailability in animal sera while

retaining the ability to bind to a Bone Morphogenetic Protein. Such a modified Noggin protein would be expected to have enhanced therapeutic properties.

5 In addition, pegylation of proteins has been shown to increase their *in vivo* potency by enhancing stability and bioavailability while minimizing immunogenicity. It is known that the properties of certain proteins can be modulated by attachment of polyethylene glycol (PEG) polymers, which increases the hydrodynamic volume of  
10 the protein and thereby slows its clearance by kidney filtration. (See, e.g. Clark, R., et al., 1996, J. Biol. Chem. 271: 21969-21977). We have generated molecules having improved pharmacokinetic properties by covalently linking polyethylene glycol (PEG) to modified human noggin.

15

The present invention also provides for pharmaceutical compositions comprising a modified Noggin molecule, as described herein and a suitable pharmaceutical carrier.

20 The active ingredient, which may comprise the modified Noggin, should be formulated in a suitable pharmaceutical carrier for systemic or local administration in vivo by any appropriate route including, but not limited to injection (e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, endoneural,  
25 perineural, intraspinal, intraventricular, intravitreal, intrathecal etc.), by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.); or by a

sustained release implant, including a cellular or tissue implant.

Depending upon the mode of administration, the active ingredient may be formulated in a liquid carrier such as saline, incorporated  
5 into liposomes, microcapsules, polymer or wax-based and controlled release preparations, or formulated into tablet, pill or capsule forms.

The concentration of the active ingredient used in the formulation  
10 will depend upon the effective dose required and the mode of administration used. The dose used should be sufficient to achieve circulating plasma concentrations of active ingredient that are efficacious. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test  
15 systems.

Practice of this invention includes preparation and uses of a diagnostic or therapeutic agent comprising a nucleotide sequence of at least about 15 DNA or RNA bases analogous to all or a portion  
20 of either Figure 14 (SEQ ID NO. 23), SEQ ID NO: 10, or SEQ ID NO: 1 or of the nucleic acid sequences contained in bacteriophages, hnog $\lambda$ -9 or hnog $\lambda$ -10. That is, noggin preparations are useful as standards in assays for noggin and in competitive-type receptor binding assays when labelled with radioiodine, enzymes, fluorophores, spin labels,  
25 and the like. Therapeutic formulations of noggin are prepared for storage by mixing noggin having the desired degree of purity with optional physiologically acceptable carriers, excipients or

stabilizers, in the form of lyophilized cake or aqueous solutions.

Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids;

5 antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins. Other components can include glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including  
10 glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or PEG.

15 Noggin may be used according to the invention as described supra. The concentration of the active ingredient used in the formulation will depend upon the effective dose required and the mode of administration used. The dose used should be sufficient to achieve circulating plasma concentrations of active ingredient that are  
20 efficacious. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

By referring to noggin, the present invention also contemplates the  
25 use of fragments, derivatives, muteins, agonists or antagonists of noggin molecules.

Noggin may be administered in any pharmaceutically acceptable carrier. The administration route may be any mode of administration known in the art, including but not limited to intravenously, intrathecally, subcutaneously, by injection into involved tissue, intraarterially, intranasally, orally, or via an  
5 implanted device. The present invention provides for pharmaceutical compositions comprising noggin in a pharmaceutically acceptable carrier.

10 Administration may result in the distribution of noggin throughout the body or in a localized area. For example, in some conditions which involve distant regions of the nervous system, intravenous or intrathecal administration of noggin may be desirable. Alternatively, and not by way of limitation, when localized regions  
15 of the nervous system are involved, local administration may be desirable. In such situations, an implant containing noggin may be placed in or near the lesioned area. Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

20 Inventive complexes comprise a ligand characterized by one or more of the SEQ ID NOS:3-7. The ligand can be bound to a protein, such as antibody. Such antibodies can be polyclonal or monoclonal. Polyclonal antibodies to noggin generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of  
25 noggin and an adjuvant. It may be useful to conjugate noggin or a fragment containing the target amino acid sequence to a protein which is immunogenic in the species to be immunized, e.g., keyhole

limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxy-succinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N} = \text{C} = \text{NR}$ .

Animals can be immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1  $\mu\text{g}$  of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally in multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later animals are bled and the serum is assayed for anti-noggin titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same noggin polypeptide, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by EB virus transformation and screening for clones expressing the desired

antibody.

In a preferred embodiment, a rat monoclonal antibody such as RP57-16, prepared after immunization of a rat with recombinant  
5 human noggin, reacts specifically with both *Xenopus* and human noggin, but not with the neurotrophins BDNF, NT-3 and NT-4.

Noggin antibodies are useful in diagnostic assays for noggin or its antibodies. In one embodiment of a receptor binding assay, an  
10 antibody composition which binds to all of a selected plurality of members of the noggin family is immobilized on an insoluble matrix, the test sample is contacted with the immobilized antibody composition in order to adsorb all noggin family members, and then the immobilized family members are contacted with a plurality of  
15 antibodies specific for each member, each of the antibodies being individually identifiable as specific for a predetermined family member, as by unique labels such as discrete fluorophores or the like. By determining the presence and/or amount of each unique label, the relative proportion and amount of each family member  
20 can be determined.

Noggin antibodies also are useful for the affinity purification of noggin from recombinant cell culture or natural sources. Noggin antibodies that do not detectably cross-react with other growth  
25 factors can be used to purify noggin free from these other family members.



Aspects of the invention will now be illustrated by the following examples.

## EXAMPLES

5

### Production of Xenopus embryos

Xenopus embryos were prepared by the protocol described by Condie and Harland (Development, 101, 93-105, 1987). Embryos were staged according to the table of Nieuwkoop and Faber ("Normal Table of Xenopus laevis"(Daubin), Amsterdam: North Holland, 1967). Ventralized embryos were produced by irradiation with a Statalinker (Stratagene), and dorsalized embryos were produced by treatment with LiCl as described by us in our paper on certain "wnt" proteins (designated "Xwnt-8"), Smith and Harland, Cell, Vol. 67, pp. 753-765 (1991) (incorporated by reference and occasionally referred to hereinafter as "S&H, supra").

10  
15

### EXAMPLE 1

#### Isolation and Sequencing of Noggin cDNA

20

The construction of the size-selected plasmid cDNA library from stage 11 LiCl-treated embryos was as follows. Sixty micrograms of poly(A) RNA from stage 11 LiCl-treated embryos was size fractionated on a 10% to 30% sucrose gradient in the presence of methylmercuric hydroxide. First strand cDNA was synthesized from 2 µg of the size-fractionated poly(A) RNAs primed with oligo(dT) oligonucleotide containing the recognition

25

site for NotI. After synthesis of the second strand, cDNAs were treated with EcoRI methylase, ligated with EcoRI linkers, digested with EcoRI and NotI, and finally ligated to 125 ng of modified pGEM-5Zf(-) (Promega). The pGEM-5Zf(-) used here was modified by the addition of an oligonucleotide into the NsiI site to create an EcoRI site. The vector was not treated with alkaline phosphatase, but the excised polylinker sequence was removed on a sepharose 4BCL column. The ligated products were used to transform XL-I Blue cells (Stratagene), and plated to give 100,000 colonies per 10 cm plate. Plasmid DNAs were isolated from plate cultures by the alkaline-lysis/polyethylene glycol precipitation protocol.

Dorsalizing activity in the library was assayed by injecting RNA transcripts made from pooled plasmid DNA. Single clones were isolated by a process of sib selection. In this procedure the plasmid library was replated on 12 plates with 10-fold fewer colonies per plate. RNA was synthesized from pooled plasmid DNAs isolated from each plate and tested for dorsalizing activity by injection into UV-ventralized embryos. Those pools with dorsalizing activity were replated and screened as described above. This process was repeated until single clones were isolated.

*In vitro* RNA synthesis, injection assay for dorsal axis rescue and sib-selections were also done, as described by us in S&H, supra.

The nucleotide sequence of both strands of the isolated noggin cDNA clone was determined by the dideoxy termination

method using modified T7 DNA polymerase (US Biochem). Deletions were prepared in sequencing templates by both restriction enzyme and exonuclease III digestion (Henikoff, Meth. Enzymol, 155, 156-165, 1987).

5

#### In vitro translation

One-half  $\mu$ g of in vitro synthesized noggin, Xwnt-8, and goosecoid mRNAs were translated in a nuclease treated rabbit reticulocyte lysate (Promega) with added  $^{35}$ S-methionine according to the manufacturer's instructions. The translation products were visualized by SDS-polyacrylamide gel electrophoresis (12% gels) followed by fluorography. Noggin protein had the molecular weight predicted by the open reading frame.

#### 15 RNA Isolation and Analysis

Total RNA was isolated from embryos and oocytes by a small scale protocol as described by Condie and Harland, supra. Dorsal lips were dissected from 30 unfixed stage 10.5 embryos and pooled for total RNA preparation. Samples containing either the total RNA equivalent of 2.5 embryos or approximately 2  $\mu$ g of poly A+ RNA were analyzed by northern blotting. Random primed DNA probes were prepared from a 1,323 bp fragment of noggin cDNA from the EcoRI site at nucleotide -83 to an EcoRV site that lies in the vector immediately 3' to the end of the cDNA.

25 RNAse protection assays were done using a protocol as detailed by Melton et al. (Nuc. Acids Res., 12, 7035-7056, 1984) with minor modifications (C. Kintner, Salk Institute, La Jolla,

California). A *Xenopus* noggin cDNA exonuclease III deletion clone (corresponding to Fig. 2A of Smith and Harland, Cell 70: 829-840 (1992) but having a deletion from the 3' end to nucleotide 383) was used as a template for synthesizing RNA probes. The template DNA  
5 was linearized by EcoRI restriction enzyme digestion and a 463 base antisense RNA incorporating <sup>32</sup>P was synthesized with T7 RNA polymerase. A 387 base antisense EFl $\alpha$  RNA probe was used as a control for amount of RNA per sample. Probes were gel purified prior to use.

10

#### In situ hybridization

After fixation and storage, the embryos were checked to ensure the blastocoel and archenteron were punctured. Care was taken to puncture the residual blastocoel of neurulae and tadpoles  
15 as well as the archenteron. Embryos were rewashed at room temperature in 100% ethanol for two hours to remove residual lipid. After hybridization, staining was allowed to develop overnight and the embryos were then fixed in Bouin's. Newly stained embryos have a high background of pink stain but most of  
20 this washes out, leaving the specific stain. Following overnight fixation, the embryos were washed well with 70% ethanol, 70% ethanol buffered with PBS and methanol. Embryos were cleared in Murray's mix and photographed with Kodak Ektar 25 film, using a  
25 Zeiss axioplan microscope (2.5 or 5x objective with 3x12B telescope to assist with focusing).

### Lineage Tracing

Lineage tracing with mRNA that encodes nuclear localized B-galactosidase was as we described in S&H, *supra*. Ventralized embryos were coinjected at the 32 cell stage with 0.5 ng B-galactosidase and 25 pg noggin $\Delta$ 5' mRNAs. Embryos were fixed and stained with X-gal at approximately stage 22.

### Results

#### Noggin cDNA Encodes a Novel Polypeptide

The 1833 nucleotide sequence of the selected clone is shown by Fig. 2A of Smith and Harland, Cell 70: 829-840 (1992) and sometimes also referred to as "clone A3." The sequence contains a single long open reading frame encoding a 222 amino acid polypeptide with a predicted molecular weight of 26 kDa. At the amino terminus, the hydrophobic stretch of amino acids suggests that the polypeptide enters the secretory pathway. There is a single potential site for N-linked glycosylation at amino acid 61 (Asn). Extensive untranslated regions are located both 5' and 3' to the reading frame (593 and 573 bp, respectively). The 3' untranslated region is particularly rich in repeated dA and dT nucleotides, and contains, in addition to a polyadenylation signal sequence located 24 bp upstream from the start of the poly A tail, a second potential polyadenylation sequence 147 bp further upstream.

Sense RNA synthesized from clone A3 with SP6 RNA polymerase was translated in a rabbit reticulocyte lysate system. The 3S-labeled products were fractionated on a 12% SDS-

polyacrylamide gel and visualized by fluorography. The major protein product had the expected molecular weight of approximately 26 kDa.

5 Comparison of the amino acid sequence of the predicted polypeptide to the National Center for Biotechnology Information BLAST network (non-redundant data base) did not identify any similar sequence. Thus, this clone encodes the new type of protein we have named "noggin" which is secreted, and which has dorsal inducing activity in *Xenopus*.

10

#### Noggin mRNA can Rescue a Complete Dorsal-Ventral Axis

Injection of noggin RNA into a single blastomere of a four cell stage UV-ventralized embryo can restore the complete spectrum of dorsal structures. The degree of axis rescue was  
15 dependent upon the amount of RNA injected, with embryos receiving low doses having only posterior dorsal structures, while embryos receiving higher doses had excess dorsal-anterior tissue. RNA transcripts from two noggin plasmids were tested. The first contained the full cDNA. The second (pNoggin $\Delta$ 5') had a deletion  
20 removing the first 513 nucleotides of the 5' untranslated region up to the EcoRI site. The resulting embryos from injection of RNA transcripts of these two plasmids, as well as *Xwnt-8* RNA for comparison, were scored according to the dorsoanterior index (DAI) scale of Rao and Elinson (Dev. Biol., 127, 64-77, 1988). In this  
25 scale, a completely ventralized embryo is scored as zero, a normal embryo is scored as 5, and the most severely dorsoanteriorized embryos, those having radial dorsoanterior structures, were scored

as 10. RNA synthesized from pNoggin $\Delta$ 5' (noggin $\Delta$ 5' mRNA) repeatedly gave a higher DAI than the equivalent amount of mRNA synthesized from the complete cDNA. The dose-dependency of axis rescue by noggin $\Delta$ 5' mRNA was very similar to that of Xwnt-8 mRNA.

UV treated embryos were also injected with a higher doses (1,000 pg) of the noggin mRNAs. Injection of this dose of noggin mRNA into one blastomere at the four cell stage resulted in embryos with very severe hyperdorsalization (DAI >7). However, most of these embryos died at the late gastrula/early neurula stage. Apparently excessively strong gastrulation movements resulted in the thinning and rupture of the blastocoel roof. We have also observed this effect with high doses of injected Xwnt-8 mRNA.

The rescue of dorsal development by both noggin $\Delta$ 5' and Xwnt-8 mRNAs followed a consistent pattern in which increasing amounts of the mRNAs lead to progressively more anterior structures being rescued. For example, embryos that received 1 pg of the RNAs had primarily the posterior and trunk dorsal structures rescued, and for the most part lacked head structures. Higher doses (10 or 100 pg) of both of the RNAs resulted in embryos with more anterior development, and many had either nearly normal or hyperdorsalized phenotypes.

#### Noggin Injected Blastomeres Act as a Nieuwkoop Center

The effect of varying the site of noggin mRNA injection was

investigated. Thirty-two cell stage UV-treated embryos were injected with either 0.5 ng of B-galactosidase mRNA alone or 0.5 ng B-galactosidase mixed with 25 pg noggin $\Delta$ 5' mRNA. Injection of noggin mRNA into blastomeres of the vegetal tier gave the most strongly dorsoanteriorized embryos. In both of the vegetal injected embryos the nuclear X-Gal staining was found almost exclusively in the endoderm (the mRNA encodes a B-galactosidase that translocates to the nucleus, allowing distinction from the diffuse background stain). One of the embryos shown was strongly hyperdorsalized (DAI approximately 7) as a result of the noggin mRNA injection, and had a severely truncated tail and enlarged head structures. Embryos were also rescued by noqgin mRNA injections into the marginal zone.

In these embryos B-galactosidase staining was observed primarily in the axial and head mesoderm. Injection of noggin mRNA into the animal pole had very little effect on axis formation. Likewise, B-galactosidase mRNA alone was without effect.

#### Noggin mRNA is Expressed Both Maternally and Zygotically

In northern blot analysis of RNA from *Xenopus* embryos two noggin mRNA species of approximate sizes 1.8 and 1.4 kb were observed. A relatively low level of noggin mRNA was detected in oocytes. By stage 11 the level of noggin mRNA was significantly higher, reflecting zygotic transcription (as opposed to the maternally deposited transcripts seen in oocytes). Noggin mRNA remained at the elevated level up to the latest stage examined (stage 45).



We expect that the primary dorsalizing RNA in our library to be elevated in LiCl-treated embryos relative to normal or UV-treated embryos. Lithium ion treatment resulted in a large increase in the amount of noggin mRNA expressed, relative to untreated embryos.

UV treatment had the opposite effect. Noggin mRNA expression was essentially undetectable in total RNA samples from these embryos. Thus, the abundance of noggin mRNA in manipulated embryos parallels the rescuing activity.

We analyzed the distribution of noggin in oocytes and cleavage stage embryos. Since the amount of maternally deposited noggin RNA is too low for in situ hybridization to detect above background, we used an RNase protection assay. Oocytes were dissected into animal and vegetal halves. No enrichment of noggin mRNA was seen in either hemisphere relative to total oocyte RNA. Four-cell stage embryos were dissected into dorsal and ventral halves, as well as animal and vegetal halves. Noggin transcripts were found to be distributed evenly between dorsal and ventral hemispheres as well as animal and vegetal hemispheres. The same result was obtained with embryos that were tilted 90° immediately following fertilization and then marked with a vital dye on their uppermost side to indicate the future dorsal side. Older (32 cell stage) blastula embryos were also dissected into dorsal-ventral and animal-vegetal halves. No enrichment of noggin mRNA in any of the hemispheres was seen relative to the total embryo. In addition, treatment did not alter the abundance of maternally deposited noggin RNA, indicating no

preferential degradation in ventral tissues. Samples with known amounts of in vitro synthesized noggin mRNA were included in the RNAase protection assay. From these and other data we estimate that there is approximately 0.1 pg of noggin mRNA per blastula stage embryo and 1 pg per gastrula stage embryo.

The localization of noggin transcripts was investigated in early gastrula stage embryos. Dorsal lips were dissected from stage 10.5 embryos. A northern blot of equal amounts of total RNA from intact embryos, dissected dorsal lips, and from the remaining embryo after dissection of the dorsal lip was hybridized with a noggin probe and then re-hybridized with an EF1a probe, as a control for amount of RNA loaded per sample. The autoradiograph of the blot showed that noggin mRNA at this stage is enriched in the dorsal lip.

#### In situ Hybridization: Zygotic Expression of Noggin in the Spemann Organizer

The localization of noggin transcripts in developing embryos was examined in greater detail using whole mount in situ hybridization. Whole fixed embryos were hybridized with digoxigenin containing RNA probes.

Hybridized RNA probe was then visualized with an alkaline phosphatase-conjugated anti-digoxigenin antibody. The specificity of hybridization seen with antisense noggin probes was tested both by hybridizing embryos with sense noggin probes, and by using two non-

overlapping antisense probes. Due both to the low level of expression, and to background staining, *noggin* mRNA could not be detected unequivocally before the late blastula stage. The increased level of *noggin* mRNA that was detected by northern blot following activation of zygotic transcription was apparent in *in situ* hybridization at stage 9 as a patch of staining cells on the dorsal side of the embryo. Viewed from the vegetal pole, this patch of cells was restricted to a sector of about 60°. A side view of the same embryo shows that the staining cells were located within the marginal zone (i.e., between the animal and vegetal poles and within the presumptive dorsal mesoderm forming region). Transcripts are largely restricted to the nucleus at this stage.

A side view of an early gastrula stage embryo 30 (approximate stage 10.5) shows specific hybridization primarily in the involuting mesoderm at the dorsal lip. A vegetal view of the same embryo (blastopore lip arrowed) shows that *noggin* mRNA is most abundant on the dorsal side, but expression extends at the lower level to the ventral side of the embryo. This method of *in situ* hybridization does not detect transcripts in the most yolky endodermal region of embryos, therefore we cannot rule out expression in more vegetal regions than those seen in the Figure. Treatments which are known to affect the size of the dorsal lip (LiCl treatment, UV irradiation) had a profound effect on the pattern of *noggin in situ* hybridization. In LiCl treated embryos the staining is intense throughout the marginal zone. UV treatment reduced the hybridization signal to low levels. This result is

consistent with amounts of noggin mRNA seen by northern blot analysis. The UV treated embryo also is a negative control for specificity of hybridization.

5       As gastrulation proceeds, noggin mRNA staining follows the involuting dorsal mesoderm, and is highest in the presumptive notochord. By the late neurula stage (approximately 18) noggin mRNA expressing cells are clearest in the most dorsal mesoderm, primarily in the notochord but also extending more anteriorly into  
10   the pre-chordal mesoderm. The anterior tip of the notochord is arrowed. During tailbud stages expression of noggin in the dorsal mesoderm declines, though expression in the notochord persists in the growing tailbud. Expression of noggin initiates at several new sites, which become progressively clearer as the tadpole  
15   matures. A discontinuous line of stained cells runs the length of the roof plate of the neural tube. Staining is also apparent in the head mesoderm, primarily in the mandibular and gill arches. We suspect that this expression corresponds to skeletogenic neural crest cells. Furthermore, subsets of these cells express homeobox  
20   genes that mark different anterior-posterior levels of the head neural crest, for example En-2 in the mandibular arch is seen by antibody staining. Cells with stellate morphology stained from noggin mRNA in the tail fin. These stellate cells are also likely to be derived from the neural crest. None of these patterns were seen  
25   with the sense probe, or with a number of other probes.

EXAMPLE 2 - Noggin cDNA Transfected into COS Cells Produces  
Active Conditioned Medium

5 For COS cells the noggin cDNA was inserted into a COS cell  
expression vector. COS cells were transfected, and medium  
harvested after allowing expression of the introduced noggin genes.  
This medium has been tested in an animal cap assay for mesoderm  
inducing or dorsalizing activity. We have tested two transfection  
10 protocols, a standard one, where cells recover and then are  
transferred to serum-free medium, and an alternate where cells  
are transferred to a defined medium lacking serum but containing  
transferrin, insulin, and BSA. Cells remain healthy in the  
supplemented medium and a cotransfected  $\beta$ -galactosidase gene  
15 gives 100 fold more activity than in the unsupplemented medium.  
The results of treating cells with these media is shown below in  
Table 1. Animal caps were taken from ventralized animals, treated  
and at the end of neurulation they were scored for elongation,  
usually a sign that notochord or neural tissues have been induced.  
20 Elongation is indicated in Table 1 by a "+" and even greater  
elongation a "++." In addition, they are scored for a molecular  
marker by Northern blotting.

As shown by the data of Table 1, the noggin cDNA has a large  
25 effect on the COS cell conditioned medium. However, noggin is  
probably interacting with something else in the medium, since  
COS-cell conditioned medium alone has some activity. It is

possible that noggin is causing the cells to secrete something that they normally would not, but the experiments do indicate that noggin is secreted and is responsible for some of the activity.

5 TABLE 1

Cos Cell Conditioned Medium: Effects on Animal Caps

		Elongation	N-CAM expression
10	Transferred to serum free medium + transferrin, BSA, and insulin		
	1. Vector only	+/-	+
15	2. Noggin cDNA	++	++
20	Transferred to serum free medium without supplements		
	1. Vector only	-	-
	2. Noggin cDNA	-	-

25

Noggin mRNA Injected into Oocytes Produces Active Secreted  
Noggin Protein

5 A second approach to studying whether protein can be  
secreted in active form is to inject oocytes with mRNA and take  
material secreted by the oocyte. A particular advantage of this  
method is that the injected mRNA is efficiently translated, and  
most of the translation of the oocyte can be taken up by the  
injected mRNA. A new protein, whose synthesis is directed by  
10 injected noggin mRNA is secreted into the medium. Noggin clearly  
synergizes with activin to produce elongated explants that express  
elevated levels of muscle actin.

Biochemical Properties of Noggin

15 Injected oocytes are injected with mRNA, and labelled with  
<sup>35</sup>S methionine. Most of the radioactive protein secreted into the  
medium is from the injected mRNA. The noggin protein, which is  
almost isotopically pure, can then be analyzed. From this analysis  
we have determined that noggin is a dimeric glycoprotein. When  
20 run under reducing conditions, and treated with N-glycanase to  
remove sugar residues, noggin migrates only slightly slower than  
its predicted molecular weight of 26 kDa. The removal of sugar  
side chains results in a loss of about 4 kDa from a starting  
apparent molecular weight of 33 kDa. When run under non-reducing  
25 conditions it migrates at double this value.

We do not yet know if the dimer of the protein is the active  
species, or if there is a proteolytically processed form which is

active. In a control experiment with activin mRNA, oocytes produce activin activity, but the bulk of the radiolabelled protein migrates as the precursor form. Only a small amount of processed protein (15 kDa) was detected. It is possible that noggin injected oocytes

5 secrete

predominantly unprocessed protein and a trace of extremely active processed protein that we have not detected. Despite the caveats, the main point from analysis of injected oocytes and transfected COS cells is that active noggin can be obtained as a freely soluble  
10 secreted polypeptide. This sets it apart from the other group of genes with dorsalizing activity, the wnts. Wnt proteins have not been available in soluble form and this has greatly hampered the analysis of their biological activities, and of the receptor that binds to them.

15

### EXAMPLE 3 - Cloning of the Mouse Noggin Homolog

It is currently impossible to eliminate zygotic noggin transcription from developing *Xenopus* embryos. In contrast, it should be possible to generate homozygous null mutations in the  
20 mouse. We have cloned the mouse noggin cDNA. This is useful to generate mutant mice. In addition to generating the probes and tools to make mutant mice, a comparison of the noggin sequences should be a useful predictor of conserved domains and functions. The C-terminal 80 amino acids are 87% identical between *Xenopus*  
25 and mouse noggin.

Mouse noggin was isolated from an embryonic cDNA library by probing with a radiolabelled frog noggin cDNA under conditions of



moderate stringency (as defined earlier). Subsequently a genomic clone was isolated by probing a genomic library with the mouse noggin cDNA

15 under conditions of high stringency (as defined, but hybridized at 42° C and washed at 50°C in 15 mM NaCl, 1.5 mM sodium citrate). The full nucleotide sequence of mouse noggin cDNA (SEQ ID NO: 10) as well as the deduced amino acid sequence (SEQ ID NO: 11) are shown in Figures 13A-13B. There are only two amino acid differences between mouse noggin and human noggin.

10

#### EXAMPLE 4 - Cloning of the Human Noggin Homolog

##### Materials and Methods

##### 15 Probe preparation

Two oligonucleotides were synthesized based on the mouse noggin sequence (supra). The sequence of the oligonucleotides is noggin 5' : 5'-CAG ATG TGG CTG TGG TCA-3' (SEQ ID NO: 18) corresponding to amino acids QMWLWS (SEQ ID NO: 19) and noggin 3' : 5'-GCAGGAACACTTACACTC-3' (SEQ ID NO: 20) corresponding to amino acids ECKCSC (SEQ ID NO: 21) of the mouse noggin protein .

The oligonucleotides were used for PCR amplification of a segment of DNA of 260 nucleotides using as a template a mouse cDNA clone prepared as set forth in Example 3. The amplified  
25 fragment had a nucleotide sequence that corresponds to nucleotides 856 through 1116 of the mouse sequence as set forth in Figure 13 (SEQ ID NO: 10). After amplification, the PCR reaction was

electrophoresed in agarose gels, the DNA band of 260 nts purified by Magic PCR (Promega), and used as template for the probe labeling reaction. The probe was labeled using a standard PCR reaction (Perkin-Elmer) on 20 ng of DNA template and 0.2 m Curie of alpha  
5 32P-dCTP (Du Pont 3000 Ci/mmol) instead of dCTP. Unincorporated label was separated from the probes on a G50 NICK column (Pharmacia). The excluded volume of the reaction contained a total of 1.8 x10<sup>8</sup> cpm.

In addition, one degenerated oligonucleotide, named noggin D,  
10 corresponding to conserved mouse and Xenopus noggin sequences, was synthesized as follows: Noggin D: 5'-GARGGIATGGTITGYAARCC-3' (SEQ ID NO: 22). Noggin D (SEQ ID NO: 22) was labeled by kinase reaction using T4 polynucleotide kinase and gamma 32P ATP. The labeled oligonucleotide was purified by  
15 NAP5 (Pharmacia) column and used for library hybridization.

#### Library screening

A human placental genomic library (Clontech Cat#HL1067J, average insert size 15 kb) in vector EMBL-3 was plated according  
20 to manufacturer specifications in NM 538 E.coli. Approximately 3 million plaques were transferred to nitrocellulose filters (BA-85 Schleicher and Schuell) in three replicas (named A, B and C) and screened according to Maniatis, et al.[Sambrook, et al., Molecular cloning a laboratory manual, CSH Lab Press, New York (1989)]. The  
25 replica filters A and C were hybridized in a buffer containing 0.5 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulphate, 1% crystalline BSA, 1 mM EDTA, 40 m g/ml denaturated salmon sperm

DNA and about  $1 \times 10^6$  cpm/ml of the PCR probe (supra). After hybridization for 12 h at 65°C, the filters were washed twice at room temperature in 2x SSC (30 mM sodium citrate, 0.3 M NaCl), 0.1% SDS and then at 65°C in 2xSSC, 0.1% SDS for 20 min and  
5 exposed to Kodak X-OMAT AR film. The filter replica B were hybridized with the labeled oligonucleotide noggin D in 6xSSC , 0.1% SDS at 51°C for 12 h followed by wash at 2xSSC, 0.1% SDS at room temperature, and in 6xSSC, 0.1% SDS at 50°C and exposed to Kodak X-OMAT AR film. Positive plaques from all replicas were  
10 isolated and purified by re-screening as above. Purified positive plaques were suspended in 500 µl SM (100 mM NaCl, 10mM MgSO<sub>4</sub> x 7H<sub>2</sub>O, 50 mM Tris HCl pH 7.5, 0.01% gelatin). 160 µl of phage suspension was mixed with 0.5 ml saturated NM538 culture, incubated for 20 min at 37°C and then inoculated into 250 ml LB  
15 containing 10 mM Mg SO<sub>4</sub>, 0.2% maltose. The cultures were incubated until cell lysis (7-8 hr) at 37°C. The phage lysates were used for phage DNA purification by the Qiagen procedure according to the manufacturers recommendations (Qiagen).

## 20 Sequencing

Sequencing was performed by using the Applied Biosystems Model 373A automatic sequencer and Applied Biosystems Taq DyeDeoxy™ Terminator Cycle Sequencing Kit.

25

## Results

Filters hybridized to the PCR mouse noggin probes (SEQ ID

NOS: 18 and 20) showed two strong signals corresponding to phage plaques named hnogλ-9 and hnogλ-10. These plaques also hybridized to degenerate oligonucleotide probe nogginD (SEQ ID NO: 22) revealed that these clones correspond to the human noggin gene. In addition, two other plaques named hnogλ-5 and hnogλ-7 produced slightly weaker signals when hybridized to the PCR probes. These clones correspond to either human noggin or related gene(s). All of the human DNA inserts can be excised from the vectors using known restriction sites as described in the literature regarding each particular library.

A 1.6 kb SacI fragment from clone hnogλ-9 containing the human noggin gene was subcloned and the nucleotide sequence determined as set forth in Figures 1A-1B. The amino acid sequence for human noggin, as deduced from the nucleotide sequence, is also set forth in Figures 1A-1B. The gene or cDNA may be expressed in various eukaryotic or prokaryotic expression systems to produce biologically active human noggin protein. It is expected that the human protein will exhibit neurotrophic activity similar to that exhibited by *Xenopus* noggin protein.

#### EXAMPLE 5 - Tissue Localization of message for human noggin

##### Materials and Methods

##### Probe preparation

Probes were prepared as set forth in Example 4. The oligos used are as follows:

SEQ ID NO: 8:

5' GAC.TCG.AGT.CGA.CAT.CGC.AGA.TGT.GGC.TGT.GGT.CAC

SEQ ID NO: 9:

5' CCA.AGC.TTC.TAG.AAT.TCG.CAG.GAA.CAC.TTA.CAC.TCG.G

- 5 (The underlined sequence represent mouse noggin sequence; the rest of the sequence are tails containing restriction sites for cloning.)

10 A DNA fragment of approximately 300 bp was obtained by PCR amplification of a mouse cDNA clone prepared as described in Example 3.

#### RNA Preparation and Northern Blots

- 15 Selected tissues were dissected from Sprague-Dawley rats and immediately frozen in liquid nitrogen. RNAs were isolated by homogenization of tissues in 3 M LiCl, 6 M urea, as described in Bothwell, et al. 1990 (Methods of Cloning and Analysis of Eukaryotic Genes, Boston, MS, Jones and Bartlett). RNAs (10 µg) were fractionated by electrophoresis through quadruplicate 1% agarose-formaldehyde gels (Bothwell, et al., 1990, Methods of Cloning and Analysis of Eukaryotic Genes, Boston, MS, Jones and Bartlett) followed by capillary transfer to nylon membranes (MagnaGraph, Micron Separations Inc.) with 10xSSC (pH7). RNAs were UV-cross-linked to the membranes by exposure to ultraviolet light (Stratalinker, Stratagen, Inc.) and hybridized at 68°C with radiolabelled probes in the presence of 0.5 M NaPO<sub>4</sub> (pH 7), 1% bovine serum albumin (fraction V, Sigma, Inc.) 7% SDS, 1 mM EDTA [Mahoudi, et al., Biotechniques 7:331-333 (1989)], 100 µg/ml
- 20
- 25

sonicated, denatured salmon sperm DNA. Filters were washed at 68°C with 3xSSC, 0.1% SDS and subjected to autoradiography for 1 day to 2 weeks with one or two intensifying screens (Cronex, DuPont) and X-ray film (AR-5, Kodak) at 70°C. Ethidium bromide staining of the gels demonstrated that equivalent levels of total RNA were being assayed for the different samples [as in Maisonpierre, et al., Science 247:1446-1451 (1990)]. RNA was prepared from the following human cell lines:

10	<u>Neuroblastoma</u>	<u>Neuroepithelioma</u>
	CHP-134	SK-N-MC
	LA-N-1	CHP-100
	LA-N-5	IARC-EWI
	IMR-32	SK-N-LO
15	SHSY5Y	SK-ES
	SKNSH	DADY
	SHEP	
	<u>Hematopoietic</u>	<u>Small Cell Lung Carcinoma</u>
20	K562	Calu 3
	U937	SKLu
	M1	NCI-H69
	TF1	SKMES
	BAF	
25	B9	
	<u>Cervical Carcinoma</u>	
		HeLa

Sympathoadrenal Precursor

MAH

Hepatoblastoma

HEPG2

Medulloblastoma

Madsen

Med

U266

5

Pheochromocytoma

PC12

10 RESULTS

We have amplified a DNA fragment from the mouse noggin plasmid, corresponding to the region conserved between *Xenopus* and mouse noggin.

15 The amplified fragment of approximately 300bp was used as probe to hybridize to northern blots, with RNAs prepared from adult and embryonic tissues, as well as from various cell lines. Noggin transcript of about 2kb in size was detected in adult rat brain, and in a cell line, SKMES, a small cell lung carcinoma.

20 Expression of noggin transcripts was examined in various tissues from rat and mouse at different stages of development and in adult. In the mouse, noggin transcripts can be detected in embryos or head from E9 to E12, as well as in newborn brain and adult brain. There was no detectable signal in peripheral tissues  
25 examined except in skeletal muscle. Abundant level of expression was also found in hippocampal astrocytes isolated from postnatal mouse.

In the rat, noggin transcripts were detectable in embryos or head from E9 to E18, as well as in brain from P1, P19 and adult brain. In the cerebellum, expression of noggin appeared to be higher in E18 and P1; in the spinal cord, expression of noggin mRNA peaked at P1. Examination of noggin expression in all of the CNS regions, especially the olfactory bulb, midbrain, hindbrain and cerebellum. In the adult, noggin mRNA could be detected in all CNS regions, especially the olfactory bulb and cerebellum. There also appeared to be low levels in the skin.

10

#### EXAMPLE 6

##### Neural Induction by Noggin

15

##### Materials and Methods

##### Preparation of Xenopus noggin CHO cell conditioned medium

Xenopus noggin CHO conditioned medium was made by selecting for stably transfected CHO cells. Dihydrofolate reductase (DHFR) deficient CHO parental cells ( J. Papkoff, Syntex Research) were transfected with a Xenopus noggin expression plasmid containing noggin in tandem with the dihydrofolate reductase gene. Growth in nucleoside free medium was used to select for successfully transfected cells. Nine colonies of transfectants were picked and grown up individually. The noggin gene in these cells was amplified by slowly increasing the dose of methotrexate, an inhibitor of DHFR. The presence of noggin

25



transcripts was first tested by Northern analysis. Subsequently, two clones, B3 and C3, were shown to secrete noggin protein, since conditioned medium from these lines was capable of dorsalizing ventral marginal zones. Furthermore, by labeling B3 cellular proteins with 35S-methionine, noggin protein could be identified as a band of about 30kD on reducing SDS-PAGE, and a band of 60kD on non-reducing SDS-PAGE indicating it forms the expected dimer. These properties matched those of the noggin protein previously produced in *Xenopus* oocytes supra, (Smith et al., Nature 361, 547-49, 1993). B3 conditioned medium was collected in a mixture of 1 part alpha MEM and 9 parts CHO-S-SFMII (Gibco-BRL). The cells were allowed to condition the medium for 3 days. Control medium from parental cells (CHO dhfr-) was collected identically. Twenty fold concentrated medium was made using Centriprep 10 concentrators, where the 20 fold change is measured by volume.

#### Purification of human noggin from COS cells

Human noggin protein was purified by a cationic exchange column. COS/M5 cells were transiently transfected with a human noggin expression plasmid, pCAE11. Cells were allowed to condition DMEM (Specialty Media) for two to three days, after which the medium was removed. Particulates from the medium were removed by a centrifugation step and subsequent passage through a 0.2um cellulose acetate filter. This cleared medium was pumped onto a MonoS (Pharmacia) column which was washed with several volumes 40mM sodium phosphate (pH 7.3), 150mM NaCl,

1mM EDTA. Proteins were then eluted in a linear gradient with 40mM sodium phosphate (pH 8.5), 1.8M NaCl, 1mM EDTA. Noggin protein elutes at 0.8M NaCl and is  $\geq 90\%$  pure by SDS-PAGE.

5     Xenopus otx isolation

          To isolate Xenopus Otx clones a tadpole head cDNA library (Hemmati-Brivanlou, et al., Development 106, 611-617, 1989) was screened with a mouse otx cDNA (S-L Ang and Rossant, Toronto) at low stringency. The clones that were picked fell into  
10    two classes. One class, which we have designated otxA, included pXOT21.2, the probe used here. By in situ hybridization, transcripts are first detected prior to gastrulation in the superficial layer on the dorsal side. During neurulation a large anterior domain expressed the gene, and includes both neural and non-neural  
15    tissues. After a decline in expression in the tailbud tadpole, the gene is reexpressed specifically in the brain and eyes.

Ventral marginal zone assay

Embryo preparation

20        Xenopus laevis embryos are fertilized and de-jellied as described (Condie and Harland, 1987. Development 101, 93-105), routinely the evening before dissections. Embryos are cultured overnight at 15°C. The vitelline membrane surrounding each developing embryo is manually removed the following morning at  
25    the late blastula stage. Until dissection, the embryos are maintained in 1/3x modified ringers in agarose coated dishes.

### Ventral marginal zone dissection

Embryos are oriented with their yolky vegetal hemisphere up so the dorsal side can be identified. The dorsal side of the early gastrula is marked by the presence of a small arc of dense pigment called the "dorsal lip" which marks the start of involution of dorsal mesoderm. The ventral marginal zone (VMZ) is found directly opposite the dorsal lip, and is dissected. Since the vitelline membrane has been removed, the embryo tends to flatten. Using a specially constructed knife made of an eyebrow, mounted onto a glass pipet with wax, two cuts are made through the flattened embryo from the top facing vegetal pole through to the animal pole. The cuts are made such that they isolate approximately 30-60 degrees of the ventral side away from more lateral tissues. A third cut which is perpendicular to the first two cuts completely isolates the ventral marginal zone tissue away from the rest of the embryo. This third cut is at the level of approximately two thirds of the radius of the embryo from the center. Prior to treatment the VMZ is washed 1x in the culture medium.

20

### Assay

Approximately between 5 to 10 VMZs are used per assay. The washed VMZs are dropped gently (trying to minimize transfer of liquid) into eppendorf tubes containing the desired treatment protein medium for assay. The VMZs are allowed to develop to the late neurula or early tailbud stage as assessed by control whole embryo development. At this time RNA is isolated from the VMZs

25

and control whole embryos as described (Condie and Harland, *ibid*). The expression of muscle actin in VMZs indicates a dorsalization event (Lettice and Slack, 1933. *Development*, 117, 263-72). RNA from each sample is run on a formaldehyde-agarose gel and blotted to gene screen. The blot is then hybridized with a *Xenopus* muscle actin probe (Dworkin-Rastl et al., 1986. *J. Embryol. exp. Morph.* 91, 153-68). Quantitation of dorsalization can be carried out by normalizing muscle actin signal to that of the ubiquitously expressed EF-1 $\alpha$  (Krieg et al., 1989. *Devl. Biol.* 133, 93-100). Quantitation is done using phosphor imaging.

#### RNase protection assay

RNase protection was carried out as described (D.A. Melton et al., *Nucleic Acids Res* 12,7035-56, 1984), with the modification that digestion was carried out at room temperature (22 C) using RNase T1 only (Calbiochem 556785) at 10 units/ml. 20-30 animal caps were harvested for each lane, of this 80% was used for neural markers and 10% for muscle actin and collagen type II. For goosecoid and brachyury 20 caps were used. Exposures ranged from 12 hours to 5 days. In all cases, films were preflashed. In cases where a marker was not expressed, the result was confirmed with greater sensitivity using phosphor imaging.

#### Results

The development of vertebrate embryos requires several inductive interactions. Mesoderm, which eventually forms tissues

such as notochord, muscle, heart, mesenchyme and blood, is induced in the equatorial region of the embryo (Nieuwkoop, Wilhelm Roux' Arch. EntwMech. Org, 162, 341-373, 1969). This inductive event is well studied, and there are several candidates for the

5 endogenous inducer(s) including members of the fibroblast growth factor(FGF) family and activin (Jessell and Melton, Cell 68, 257-70 1992; Sive, Genes Dev 7, 1-12, 1993) and TGFb family (Asashima, et al., Roux's Arch. Dev. Biol. 198, 330-335, 1990; Asashima, et al., Naturwissenschaften 77, 8, 389-91, 1990; Green

10 and Smith, Nature 347, 391-394, 1990; Smith, et al., Nature 345, 6277, 729-31, 1990; Thomsen, et al., Cell 63, 485-493, 1990; van, et al., Nature 345, 6277, 732-4, 1990). The use of dominant negative receptors for both FGF (Amaya, et al., Cell 66, 257-270, 1991) and activin (Hemmati-Brivanlou and Melton, Nature 359,

15 609-614, 1992) in *Xenopus* embryos strongly suggests that the signaling pathways activated by these molecules are essential for proper mesoderm formation. Molecules such as wnts (Christian, et al., Development 111, 1045-1055, 1991; McMahon and Moon, Cell 58, 1075-84, 1989; Smith and Harland, Cell 67, 753-765, 1991;

20 Sokol, et al., Cell 67, 741-752, 1991) and noggin (Smith, et al., Nature 361, 547-49, 1993) modify the kinds of mesoderm made without inducing mesoderm directly.

In a subsequent induction, the dorsal mesoderm of the Spemann organizer signals nearby lateral mesoderm to take on a

25 more dorsal fate (Dale and Slack, Development 100, 2, 279-95, 1987; Lettice and Slack, Development, 117, 263-271, 1993; Spemann and Mangold, Arch. mikrosk. Anat. EntwMech. 100, 599-

638, 1924; Stewart and Gerhart, Development 109, 363-372, 1990). The only known factor which is expressed in the organizer and can mimic its dorsalizing activity is noggin.

Dorsal mesoderm of the Spemann organizer also signals nearby ectoderm to become neural tissue. Neural induction by dorsal mesoderm has been demonstrated in amphibians (Dixon and Kintner, Development 106, 749-757, 1989; Doniach, et al., Science 257, 5069, 542-5, 1992; Hamburger, The Heritage of Experimental Embryology: Hans Spemann and the Organizer, 1988; Kintner and Melton, Development 99, 311-25, 1987; Spemann, Arch. mikrosk. Anat. EntwMech. 100, 599-638, 1938), birds (Kintner and Dodd, Development 113, 1495-1506, 1991; Tsung, et al., Acta Biol exp Sinica 10, 69-80, 1965), and recently in mice (Ang and Rossant, Development 118, 139-149, 1993). Despite decades of effort, little is known about the molecular nature of the factors responsible for this induction. Among known inducers, activin can promote formation of neural tissue, but this is due to a secondary induction by the dorsal mesoderm that activin induces (Green, et al., Development 108, 1, 173-83, 1990; Green and Smith, Nature 347, 391-394, 1990; Kintner and Dodd, Development 113, 1495-1506, 1991). Thus, activin cannot promote formation of neural tissue when added to gastrula ectoderm; however, such ectoderm remains competent to be neuralized by dorsal mesoderm until the end of gastrulation (Sharpe and Gurdon, Development 109, 765-74, 1990).

Direct Neural Induction by Noggin

Candidates for the endogenous inducer are expected to induce neural tissue in the absence of dorsal mesoderm. Competent animal cap ectoderm from late blastula stage embryos (St9) was used to test noggin's neural inducing capacity. Xenopus noggin protein conditioned medium was collected from stably transfected CHO cells and twenty fold concentrated medium was used to treat St 9 animal caps. Markers used in an RNase protection assay were N-CAM (Jacobson and Rutishauser, Developmental Biology 116, 524-31, 1986; Kintner and Melton, Development 99, 311-25, 1987), a neural cell adhesion molecule, a neural specific isoform of  $\beta$ -tubulin (Good, et al. Nucleic Acids Res 17, 8000, 1989; Good, et al., Dev Biol 137, 414-8, 1990; Richter, et al., Proc Natl Acad Sci USA 85, 8086-90, 1988) that is expressed in the hind brain and spinal cord, and XIF3, a neurally expressed intermediate filament gene (Sharpe, et al., Development 107, 701-14, 1989) to assay for neural induction. All these markers are restricted to neural tissue, however, only NCAM is expressed throughout the nervous system. We found that Xenopus-noggin conditioned medium induces high levels of N-CAM and XIF3 expression[Fig. 2.; lane8] in treated animal caps, without inducing muscle actin(lane 13) (Dworkin-Rastl, et al., J. Embryol. exp. Morph. 91, 153-168, 1986; Mohun, et al., Nature 311, 716-721, 1984). Control CHO cell medium induces neither muscle nor neural tissues (lanes 7,12). St 9 activin treated animal caps express muscle actin(lane11) and all three neural markers(lane 6), demonstrating activin's ability to generate neural tissue indirectly. It is interesting to note that

noggin induces very little, if any b-tubulin expression, while inducing high levels of N-CAM, but activin induction has nearly the converse effect.

To determine whether noggin protein is sufficient to induce neural tissue, COS cells were transfected with pCAE11, a human  
5    noggin expression plasmid, and the conditioned medium was purified by cation exchange chromatography resulting in noggin preparations that were 90% pure [Fig. 3.]. Such purified human  
10   noggin protein is also able to induce neural tissue in animal caps [Fig. 4a., see below].

We have shown that noggin does not induce muscle in late blastula stage animal caps, however, it is possible that noggin induces other types of dorsal mesoderm. To address this concern, we asked whether noggin could induce the expression of the early  
15   mesoderm markers goosecoid (Blumberg, et al., Science 253, 194-6, 1991; Cho, et al., Cell 67, 1111-20, 1991), a marker of organizer tissue and subsequently head mesoderm or X-brachyury (Smith, et al., Cell 67, 79-87, 1991), which appears to be  
20   expressed in all mesodermal precursors early, and subsequently is expressed in posterior mesoderm and notochord. Animal caps were treated at stage 9 and collected at stage 11, when expression of goosecoid and brachyury in the normal embryo is high. Neither  
25   marker is turned on by purified human noggin (Fig. 4b. lane 5) at a dose with demonstrated neural inducing activity (Fig 6 lane15); in contrast animal caps treated in the same fashion with activin show both goosecoid and X-bra expression (Fig. 4b. lane 4) as expected for this mesoderm inducing factor (Cho, et al., Cell 67, 1111-20,



1991; Smith, et al., Cell 67, 79-87, 1991). Untreated animal caps show no expression of these mesodermal markers (lane 3), and RNA levels in the collected animal caps are shown to be comparable using EF-1a levels (Krieg, et al., Dev Biol 133, 93-100, 1989).

5        Since purified human noggin is capable of driving neural induction, no additional factors which may have been present in the crude conditioned medium are required. Furthermore, Xenopus and human noggin, with 80% amino acid identity, can both act to induce neural tissue in Xenopus, suggesting a conserved function for these  
10       two proteins. However, for noggin to be a candidate endogenous neural inducer it must be able to induce neural tissue at a stage when neural induction occurs in normal whole embryos. It is unclear when the first instructive signals are sent from dorsal mesoderm to ectoderm in embryos. However, it is known that by  
15       early gastrula stages, dorsal ectoderm has already been specified to become neural tissue (Jones and Woodland, Development 107, 785-91, 1989). The neural inducing signal is therefore likely to start before this stage. The latest stage at which animal caps have been shown to be competent to respond to neural-inducing  
20       mesoderm is the early neurula (St13-14) (Sharpe and Gurdon, Development 109, 765-74, 1990). Thus, a candidate endogenous neural inducer must be able to induce neural tissue from gastrula stage competent ectoderm.

#### 25       Neural Induction at the Gastrula Stage

      In order to assess the competence of ectoderm to respond to noggin we treated animal caps taken from blastula (St8), late

blastula (St9), early gastrula (St10) and ventral animal caps from mid-gastrula (St10.5) stage embryos with purified human noggin[Fig. 2.]. We also treated similarly staged animal caps with activin to demonstrate its mesoderm inducing and secondary neural inducing activities, and to contrast activin's effects with those of 5 noggin[Fig. 4a.]. Activin treated animal caps show neural induction only in conjunction with induction of dorsal mesoderm, such as muscle and notochord (lanes 3,6,9). In a number of experiments, we confirmed that activin's ability to induce dorsal mesoderm, and 10 consequently neural tissue, declines rapidly at the gastrula stage (lane 12) (Green, et al., Development 108, 173-83, 1990; Kintner and Dodd, Development 113, 1495-1506, 1991). In the experiment shown here a larger than usual dose of activin was given. Under these conditions, only a small amount of neural tissue is made, 15 perhaps because so much mesoderm is induced that there is not much competent ectoderm left in the explant to be neuralized. In contrast noggin can induce neural tissue in animal caps taken from all of these stages without inducing the notochord and somite marker, collagen type II (Amaya, et al., Development 118, 477-87, 20 1993; Bieker and Yazdani-Buicky, J Histochem Cytochem 40, 1117-20, 1992), or muscle actin (lanes 4,7,10,13). This gives additional support to the proposal that noggin is a direct neural inducer, since it can act in the absence of both early and late mesoderm markers. Furthermore, we have shown that noggin can 25 induce neural tissue in competent ectoderm at a time when mesoderm inducers are inactive.

In some experiments, noggin addition to gastrula (but not

blastula) animal caps resulted in induction of muscle. This occurred at stages when activin could no longer induce muscle. We interpret this as a result of a dorsalizing action by noggin on tissues that have received a weak mesoderm-inducing signal. The mesoderm-inducing signal which spreads into the gastrula animal cap is not enough to induce mesoderm, but in the presence of Xwnt-8 or noggin, muscle is formed. One interesting corollary of the induction of muscle is that the kinds of neural tissue seen in the explant are modified. Induction in explants that contain no muscle usually yields N-CAM expression, but if muscle is present, expression of both N-CAM and b-tubulin is seen. This phenomenon is demonstrated in the secondary neural induction by activin in St. 9 animal caps[Fig. 2.] and in the comparison of neural tissue induced by noggin in ventral marginal zones versus animal caps [Fig. 6.]. In the ventral marginal zones and animal caps in which muscle is present, both N-CAM and b-tubulin are expressed, whereas induced animal caps without muscle, show only N-CAM expression.

20 Neural induction after injection of DNA coding for noggin

To confirm our conclusions using a different experimental approach, we have directed noggin expression to gastrula stage animal caps by injecting the plasmid pCSKA-noggin into the animal pole of a one cell stage embryo. This plasmid, in which noggin is under the control of the cytoskeletal actin promoter, turns on the expression of noggin mRNA at the onset of gastrulation (Smith, et al., Nature 361, 547-49, 1993). At the blastula stage, the animal

caps are dissected and then matured to tailbud stages for molecular analysis. Animal caps injected with the noggin plasmid show expression of N-CAM in the absence of muscle or notochord markers (Fig. 4c. lane 2). A control plasmid directing the  
5 expression of lac Z showed no neural or mesodermal induction as expected (lane 1). This experiment demonstrates that ectopic noggin expression can directly induce neural tissue in gastrula stage ectoderm, a stage when neural induction is taking place in whole embryos.

10

Differences in competence between dorsal and ventral animal caps.

Animal caps taken from the dorsal side of gastrula stage embryos show greater competence to form neural tissue than ventral animal caps (Otte and Moon, Cell 68, 1021-29, 1992;  
15 Sharpe, et al., Cell 50, 749-58, 1987), when involuted anterior mesoderm is used as the inducer. This type of mesoderm, however, has weaker inducing capacity than the rest of the involuted mesoderm (Sive, et al., Cell 58, 171-180, 1989). Furthermore, the ventral side of an embryo can support the formation of a  
20 complete secondary axis when the organizer is placed on that side (Gimlich and Cooke, Nature 306, 471-3, 1983; Smith and Slack, J. Embryol. Exp. Morph. 78, 299-317, 1983; Spemann, Arch. mikroskop. Anat. EntwMech. 100, 599-638, 1938), indicating that there is no qualitative difference in competence. Thus, while a  
25 weak inducer might unmask slight differences in competence of the ectoderm, it has been suggested that a robust neural inducer would show little difference in its effects on dorsal and ventral

ectoderm (Servetnick and Grainger, Development 112, 177-88, 1991). Therefore we tested noggin's effects on dorsal and ventral ectoderm from the early gastrula. No difference in N-CAM expression is detected (Fig. 5, lanes 4,6), while the ventral animal caps treated with noggin show a greater amount of muscle actin expression (presumably through dorsalization of tissues that received a low-grade mesoderm induction). Activin treated dorsal caps show induction of roughly the same level of muscle actin expression (lane 5) as the ventral noggin treated caps, however, 5  
10 activin treatment did not induce detectable neural specific transcripts (lanes 3,5). This indicates that muscle tissue induced at this stage is not sufficient to secondarily induce neural tissue, and that noggin must be present to induce neural tissue.

We conclude that there is no dorsal-ventral difference in 15  
noggin mediated neural induction, suggesting that noggin behaves like the robust neural inducing signal of the Spemann organizer, not like the weaker signal from early anterior mesoderm.

#### Dose Dependence

20 To determine what levels of noggin protein are required for neural inducing activity, we carried out a dose response experiment. In addition to determining the doses required for neural induction in animal caps, we have also carried out a dose response of the dorsalization of ventral marginal zones in order to 25  
compare the doses required for these two types of inductions. Stage 9 animal caps or St. 10.5 VMZ were treated with purified human noggin, and N-CAM and  $\beta$ -tubulin were used to assay neural

induction, while muscle actin was used as a marker of dorsal mesoderm. This experiment shows that neural induction occurs at a dose of 1µg/ml, which is a twenty fold higher dose than required for dorsalization of VMZ [Fig. 5]. There are several observations  
5 that may account for the apparently high dose requirement. First, to get a maximal neural response from dorsal mesoderm, the tissues must be left in contact through most of neurulation (Sharpe and Gurdon, Development 109, 765-74, 1990); in contrast, the animal caps treated with noggin close up  
10 rapidly, this inhibits factor access, and consequently they receive only a brief effective dose. Second, it is likely that noggin is not the only neural inducer active in the embryo; it has been shown in a variety of amphibians that the somites (Hemmati-Brivanlou, et al., Science 250, 800-802, 1990; Jones and Woodland, Development  
15 107, 785-91, 1989) and the neural plate have neural inducing activity (Hamburger, The Heritage of Experimental Embryology: Hans Spemann and the Organizer, 1988; Servetnick and Grainger, Dev Biol 147, 73-82, 1991) and noggin transcripts are not detected there. Thus it is plausible that noggin is one of several  
20 neural-inducing activities. In this connection it is worth noting that noggin is equally potent in inducing neural tissue in ventral marginal zones as in dorsalizing them to generate muscle. Numerous other experiments (see Fig. 5) show that induction of a similar amount of muscle at this stage by activin does not result in  
25 neural induction. Fourth, it may be that only a small fraction of the purified protein is active, and that the experiment overestimates the amount of protein needed for neural induction. Finally, it is

possible that the accessibility of exogenously added soluble noggin is significantly lower than noggin protein being secreted endogenously.

## 5     Patterning

Embryonic neural tissue develops an anteroposterior (A-P) pattern, with various brain structures, eyes, and the spinal cord. It is thought that A-P neural pattern requires the presence of dorsal mesoderm, whether it be adjacent to the responding ectoderm in a planar configuration (Dixon and Kintner, Development 106, 749-757, 1989; Doniach, et al., Science 257, 542-5, 1992; Kintner and Melton, Development 99, 311-25, 1987; Ruiz i Altaba, Development 108,595-604, 1990) , or directly beneath it in a vertical interaction (Dixon and Kintner, Development 106, 749-757, 15 1989) (Hemmati-Brivanlou, et al., Science 250, 800-802, 1990; Sharpe and Gurdon, Development 109, 765-74, 1990; Sive, et al., Cell 58, 171-180, 1989) . Both of these types of interactions occur in normal development, and both probably contribute to the resulting pattern. To determine if noggin induces patterned neural tissue, and if so, what neural regions are represented, we used 20 Xenopus otx as a marker of forebrain and mid brain; En-2 (Hemmati-Brivanlou, et al., Development 111, 715-724, 1991) as a marker of the mid brain-hind brain boundary, and Krox-20 (Wilkinson, et al., Nature 337, 461-4, 1989) as a marker of 25 the third and fifth rhombomeres of the hind brain in in situ hybridization (Harland, Methods in Cell Biology, 36, 675-685, 1991) . Antibodies directed against XIHbox 6 (Wright, et al.,

Development 109, 225-34, 1990) mark posterior hind brain and spinal cord structures. Prior to the use of these markers, we observed the formation of cement glands in noggin treated animal caps. Since cement glands are induced organs of ectodermal origin found anterior to the neural plate, this result suggests that noggin induces anterior structures. In situ hybridization confirms this by showing the presence of a cement gland specific transcript, XAG-1 (Sive, et al., Cell 58, 171-180, 1989) in noggin treated animal caps, but not in control treated animal caps[Fig. 7A-L.]. In situ hybridization with the region specific neural markers[Fig. 7A-L.] show that noggin induces forebrain type tissue as seen by the expression of otx in noggin treated animal caps. We have not detected En-2, Krox20, or XIHbox, suggesting that these more posterior markers are not induced by noggin.

15

#### Expression of neural antigens

We have demonstrated that noggin directly induces the expression of neural specific transcripts. A further demonstration is to use antibodies against neural specific antigens to show that the noggin induced tissue is phenotypically neural. To this end, we have treated animal cap tissue with noggin and cultured them to a late stage (St 35) for antibody staining. We have used the 6F11 anti-N-CAM antibody, which stains the entire neural tube of a normal embryo. Noggin treated animal caps express this antigen [Fig. 7A-L.] while control untreated animal caps do not. This indicates that noggin can induce the production of neural specific proteins in treated animal caps. We have failed to detect the

25



expression of numerous other antigens that are characteristic of various subclasses of differentiated neural cells. These included 2G9, which stains most neural tissue, including peripheral neurons, Tor 24.55, which stains sensory neurons, and Tor 23, which stains  
5 a variety of neurons including motor neurons.

#### EXAMPLE 7

Production of recombinant human noggin from E. coli and  
10 baculovirus

#### Materials and Methods

#### Genetic Engineering and Cell Culture

15 A lactose inducible expression plasmid was constructed by replacing the Swa1/Bsm1 region of pRPN40 (Masiakowski et al , J. Neurochem. 57, 1003-1012, 1991 ) with the Swa1/Bsm1 region of the human noggin gene obtained by PCR and spanned by the same restriction sites, resulting in plasmid pRG301. pRG301 is a high  
20 copy number kanamycin resistant plasmid derived from pBR322 with the human noggin gene under the control of the lacUV5 promoter. A plasmid containing the high copy number kanamycin resistant gene was deposited with the Agricultural Research Collection (NRRL), Peoria, Illinois, and bears accession number B-  
25 18600. This plasmid was described in United States Patent Application Serial No. 07/478,338, which is incorporated by reference herein in its entirety. E. coli W3110lacIq cells

transformed with pRG301 were grown at 37°C, induced with lactose, harvested by centrifugation, washed once with 100 mM Tris-HCl, 50 mM EDTA pH 8 and stored frozen, essentially as described (Masiakowski et al, *ibid.* ).

5

#### Recovery from inclusion bodies

E. coli cell paste (32 g) was suspended in ten volumes (v/w) of 50 mM TrisHCl-pH 8.0-5 mM EDTA, lysed in a French Press at 8,000 psi and 8°C and centrifuged at 8,000xg for 30 min at 4°C.

10 The pellet containing noggin was suspended in the original volume of 2 M urea-20 mM TrisHCl, pH 8.0 and stirred for 30 min. The suspension was centrifuged at 8,000xg at 4°C for 30 min and the pellet consisting mostly of inclusion bodies (IB) was suspended in 20 volumes (v/w) of 6 M guanidine HCl, 50 mM TrisHCl, 1 mM EDTA,

15 50 mM DTT and stirred for one hour at room temperature. After centrifugation at 8,000xg for 30 min, the supernatant containing 0.45-0.50 g denatured and reduced noggin was diafiltered against 10 volumes of 6 M urea-50 mM sodium acetate pH 4.5-1 mM EDTA-0.1 mM DTT using Omega 10,000 MW cut-off membranes. The

20 diafiltrate containing 0.4-0.44 g noggin was loaded at a flow rate of 30 ml/min onto a 2.6 x 10 cm column of S-Sepharose (Pharmacia), equilibrated in 6 M urea-50 mM sodium acetate-1 mM EDTA-0.1 mM DTT pH 4.5 . The column was first washed with the same buffer and then with a one liter gradient (0-1M NaCl) at a

25 flow rate of 30 ml/min. Fractions containing noggin were identified by gel electrophoresis and pooled. The yield was 0.2-0.25 g noggin.

### Refolding

The denatured and reduced noggin solution was adjusted to .05-.2 mg/ml protein concentration and brought to 1.5-2.5 M  
5 guanidineHCl-0.1 M TrisHCl pH 8.0-0.1 mM EDTA-0.2-2 mM reduced glutathione-0.02-0.2 mM oxidized glutathione (preferably at a ratio of 10:1 reduced to oxidized glutathione) at 4°C under slow stirring. After 24-72 hours, two refolded noggin isoforms were identified by RP-HPLC chromatography (Fig. 8). The refolded noggin solution  
10 was diafiltered against 20 volumes of 0.05 M sodium acetate pH 4.5, brought to 50 mM potassium phosphate pH 7.2 and stirred slowly at 4°C for 1 hour minimum. Misfolded noggin precipitated and was removed by centrifugation for 30 min at 8,000 xg.

### 15 Reverse phase HPLC chromatography.

Refolded noggin can be purified by chromatography on a 12 mm C8, 1 x 25 cm Dynamax 300 A column equilibrated in solvent A (0.1% TFA in water). After loading, the column was washed with  
20 solvent A and was developed at a flow rate of 4 ml/min according to the following protocol: (a) 10 min isocratically at 70% of solvent A, 30 % of solvent B (0.1% TFA in acetonitrile); 30 min linear gradient to 60 % solvent B and 40 % solvent A. Correctly refolded noggin elutes earlier at 44%-46% solvent B. The yield was 0.07-0.1 g noggin.

25

### Production of human noggin in Baculovirus cell culture.

The SF21 line of *Spodoptera frugiperda* was routinely

maintained as cell monolayers in Grace's Insect Cell medium supplemented with lactalbumin hydrolysate and yeastolate (Gibco). This medium completed with 10% v/v heat-inactivated fetal calf serum (Irvine Scientific) is identified as TMNFH-10. Cells were  
5 also cultured in serum-free medium (SF-900-II; Gibco) after adaptation. Suspension cultures in either medium were raised in microcarrier culture flasks (Bellco) using a stirring speed of 80 rpm. All cultures were maintained at >96 % viability, as judged by trypan blue exclusion.

10

#### Construction of recombinant baculovirus.

Sequences corresponding to human noggin were excised as a 5'-BamH1—Pst1-3' fragment from an expression plasmid containing  
15 the human noggin gene. This fragment was inserted into BamH1—Pst1 digested pVL1393 (Invitrogen). The resulting plasmid, pTR 1009, has the human noggin sequence immediately downstream of the polyhedrin promoter of Autographa californica Multiple Nuclear Polyhedrosis Virus (AcMNPV), and this promoter-  
20 heterologous gene fusion is flanked in turn by recombination targets derived from the AcMNPV polyhedrin region. Recombinant plasmid DNA was purified by alkaline lysis and CsCl centrifugation. SF21 cells were co-transfected with plasmid and viral DNA by the following method: Plasmid DNA (3 mg) was mixed with 0.5 mg  
25 linearized, deleted viral DNA (Baculo Gold™, Pharmingen), and precipitated with ethanol. Dried DNA was then resuspended in water (50 ml), mixed with 1.5ml Grace's medium, and 30 ml

Lipofectin™ cationic liposomes (BRL). The DNA-liposome mixture was vortexed, allowed to stand at room temperature for 15 minutes and added dropwise to a monolayer of SF21 cells (2x10<sup>6</sup> cells/60 mm plate). After incubation at 27°C for four hours, 2 ml  
5 TMNFH-10 was added and the culture returned to incubation for 5 days. Tissue culture medium was harvested and used as a source of virus for plaque isolation.

Recombinant virus was isolated by multiple rounds of plaque purification on SF21 cells. Diluted virus (0.5 ml) was adsorbed to  
10 cell monolayers (2x10<sup>6</sup> cells/60 mm plate) for a period of one hour at 27°C, aspirated, and virus plaques were allowed to develop with an overlay of 0.5% agarose in TMNFH-10 medium for a period of 6 days. Virus plaques were picked after microscopic inspection, and eluted into 2ml SF900-II medium. Virus stocks were amplified by  
15 low multiplicity (0.1 pfu/cell) infection. Virus clones expressing noggin were identified by metabolic labeling of infected cultures with 35S-methionine and 35S-cysteine and analysing total labeled protein by polyacrylamide gel electrophoresis and autoradiography.

A labeled protein of the expected apparent Mr of 20,000-30,000  
20 was detected by this method in candidate clones but not in control cultures.

#### Expression and purification of baculovirus-derived noggin.

SF21 cells were cultured in suspension flasks to a density of  
25 approximately 1.8x10<sup>6</sup>/ml in SF900-II medium. Cultures (500 ml) were collected by centrifugation at 1000xg for 10 min and resuspended in 20ml of growth medium containing 5-10 pfu/cell

recombinant virus. Virus was allowed to adsorb for 1 hour at room temperature with gentle mixing. Infected cells were then diluted to their original volume with fresh growth medium, and incubated at 27°C for 3 days. Cells and debris were subsequently clarified  
5 from the growth medium by centrifugation at 1000x g for 20 min.

Cell supernatants were brought to pH 8.0, passed through a 0.45 mm Millipak 60 filter and applied to a Fast S column that had been equilibrated in 25 mM HEPES pH 8.0. The column was washed with the same buffer and developed with a linear NaCl gradient to  
10 remove other medium components. Noggin eluted from this column at 1 M NaCl.

## Results

### Characterization of human noggin produced in E. coli and in 15 baculovirus

Reverse-phase HPLC chromatography shows that recombinant noggin refolded and purified from E. coli elutes in a single sharp peak, indicating the presence of one predominant isoform (Fig. 9). Electrophoresis on 15 % polyacrylamide-SDS-reducing gels shows  
20 that noggin from either E. coli or insect cells is better than 95 % pure and migrates in a single band corresponding to a protein of 20-30 kD. Noggin from insect cells shows slightly slower mobility, apparently due to additional mass from N-linked glycosylation at the single consensus site (Fig. 10). Treatment  
25 with Endo F converts the mobility of insect-produced noggin to that of the bacterially produced protein.

In the absence of reducing agents, noggin produced either in E.coli or in baculovirus behaves as a disulfide-linked oligomeric protein (Fig. 10). However, by gel filtration analysis and mass spectroscopy noggin is primarily a dimeric protein.

5

Circular dichroism studies show that recombinant noggin refolded and purified from E. coli as well as noggin purified from insect cells have very similar conformations (Fig. 11). Secondary structure determined by this method indicates that noggin consists of 48% alpha-helix, 0 % beta-structure, and 52 % random coil.

10

#### Biological Activity of human noggin produced in E. coli and in baculovirus

Biological activity of human noggin produced in E. coli or in baculovirus was determined by assay of muscle actin expression in the ventral marginal zone assay, as described supra. Results shown in Fig. 12 indicate a positive dose response for induction of muscle actin mRNA in VMZ exposed to either bacterially produced human noggin, or baculovirus produced human noggin.

20

#### EXAMPLE 8 - Production and characterization of rat monoclonal antibody RP57-16 reactive with human noggin.

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##### Materials and Methods

##### 25 Production of antibody

RP57-16 rat monoclonal antibody reactive with recombinant human and Xenopus noggin was produced by the immunization of a

female Lewis rat with four 35 µg injections of purified recombinant human noggin (produced in *E. coli*) over a two month period. For the initial immunization, the protein was injected in the rear foot pad in Freund's complete adjuvant. Subsequent  
5 injections were given in the same foot pad in Freund's incomplete adjuvant. The rat was euthanized 3 days after the fourth injection.

Lymph node cells from the immunized rat were mixed with SP2/0-E.O. mouse myeloma cells at a ratio of 2:1. After centrifugation, the cell mixture was resuspended in 0.25 ml of 42%  
10 (w/v) PEG 3350 (Baker) in phosphate-buffer-saline with 10% (v/v) dimethylsulfoxide (Sigma) for a total of 3 minutes in a 37°C water bath. Cells were plated at a density of  $5 \times 10^4$  lymphocytes per well in 96-well plates (Falcon 3072) in DMEM/F-12 (Mediatech, Inc.) containing 10% FBS (supplemented with streptomycin,  
15 penicillin, pyruvate, and glutamine) and HMGIT ( $1.6 \times 10^{-3}$  M thymidine,  $4.0 \times 10^{-4}$  methotrexate,  $1.3 \times 10^{-3}$  sodium bicarbonate and  $1.0 \times 10^{-2}$  hypoxanthine). After 10 days in culture, supernatants were harvested and assayed for antibody activity against recombinant human noggin by indirect ELISA. Supernatant  
20 from COS-M5 cells transfected with the plasmid containing the human noggin gene was air dried overnight in Probind 96-well assay plates (Falcon 3915). Non-specific binding was eliminated by 2 hour incubation at ambient temperature with PBS/1% BSA (Sigma). Plates were washed 2 times with PBS/0.02 % Tween 20.  
25 Culture supernatants were then added and incubated at ambient temperature for 1 hour. Plates were washed 4 times with PBS/0.02 % Tween 20. Secondary antibody, Goat anti-Rat IgG (H+L)



alkaline phosphatase conjugate(Caltag) diluted 1:2000 in PBS/1% BSA was added to each well and the plates incubated at ambient temperature for 1 hour. Plates were again washed 4 times with PBS/0.02 % Tween 20. Antibody binding was visualized by 1 hour incubation at ambient temperature in the dark with pNPP (p-nitrophenyl phosphate, Sigma) 1 mg/ml in diethanolamine buffer, pH 9.8. The reaction was stopped by the addition of an equal volume of 100 mM EDTA. Absorbance was read at 405 nm on a Thermomax Microplate Reader (Molecular Devices). A reaction was considered positive if the absorbance was 2 times that of the negative control (diluent alone followed by secondary antibody and substrate). Positive clones were expanded and culture supernatant containing monoclonal antibody was collected for specificity analysis.

RP57-16 was cloned in soft agar. Cloned hybrid cells were expanded in DMEM/F-12 (Mediatech, Inc.) containing 10% FBS (supplemented with streptomycin, penicillin, pyruvate, and glutamine). Supernatant containing antibody was aliquoted and stored at -70°C until use.

#### Specificity Analysis

##### ELISA

100 ng of purified recombinant human noggin, Xenopus noggin, BDNF, NT-3, and NT4 protein was individually passively adsorbed to Probind 96-well assay plates by overnight incubation at 4°C in 50 mM bicarbonate buffer, pH 9.6. BDNF, NT-3 and NT-4 were used to assess non-specific binding of rat monoclonal antibody RP57-

16. Supernatants from COS-M5 cells transfected with either the plasmid containing the human noggin gene or the plasmid containing the flg C-terminal tagged Xenopus noggin gene were air dried to Probind 96-well plates overnight. Non-specific binding was  
5 eliminated by 2 hour incubation at ambient temperature with PBS/1% BSA (Sigma). Plates were washed 2 times with PBS/0.02 % Tween 20. Undiluted RP57-16 was added and incubated at ambient temperature for 1 hour. Plates were washed 4 times with PBS/0.02 % Tween 20. Secondary antibody, Goat anti-Rat IgG (H+L)  
10 alkaline phosphatase conjugate(Caltag) diluted 1:2000 in PBS/1% BSA was added to each well and the plates incubated at ambient temperature for 1 hour. Plates were again washed 4 times with PBS/0.02 % Tween 20. Antibody binding was visualized by 1 hour incubation at ambient temperature in the dark with pNPP (p-  
15 nitrophenyl phosphate, Sigma) 1 mg/ml in diethanolamine buffer, pH 9.8. The reaction was stopped by the addition of an equal volume of 100 mM EDTA. Absorbance was read at 405 nm on a Thermomax Microplate Reader (Molecular Devices). A reaction was  
20 considered positive if the absorbance was 2 times that of the negative control (diluent alone followed by secondary antibody and substrate).

#### Electrophoresis and Western Blotting

Rat monoclonal antibody RP57-16 was also analyzed by  
25 Western blotting. 50 ng of recombinant human noggin, non-reduced and reduced, were electrophoresed on 12.5% SDS-polyacrylamide gels and electroblotted on nitrocellulose membranes. Membranes

were blocked with PBS/1% Casein/0.1% Tween 20, and then incubated for 2 hours with undiluted RP57-16 culture supernatant. Following 4 washes in PBS/0.02% Tween 20, the membranes were incubated with a 1:5000 dilution of Goat anti-Rat IgG (H+L) horseradish peroxidase conjugate (Pelfreeze) in PBS/1% BSA/0.1% Tween 20. Membranes were washed 4 times with PBS/0.02% Tween 20. Proteins were visualized with ECL Western Blotting Reagents (Amersham) according to the manufacturer's instructions. Membranes were then exposed to XAR 5 Scientific Imaging film (Kodak) for 5 seconds.

## RESULTS

Rat monoclonal antibody RP57-16 reacts with both recombinant human and *Xenopus* noggin and with recombinant human noggin produced in *E. coli*, in insect cells, and in COS-M5 cells. The antibody does not react with the neurotrophins BDNF, NT-3 and NT-4. Western blotting showed that the antibody detects both reduced and non-reduced protein.

## EXAMPLE 9 - CREATION OF NOGGIN DELETION MUTEINS

The subject invention further concerns the discovery that native human noggin can be modified to create new compounds with highly desirable characteristics.

The basic region of Xenopus noggin corresponds to amino acids 123-135 (KKHRLSKKLRRKL) of the molecule. (See Smith, W.C. and Harland, R.M. Cell 70: 829-840 (1992) for sequence). Based upon  
5 what was known about the TGF- $\beta$  family of molecules, the basic region appeared to be a possible processing site. To better understand the function of the basic region, constructs of Xenopus noggin were created that deleted the highly conserved basic region ( $\Delta$ 123-135) or a larger portion of the peptide including the basic  
10 region ( $\Delta$  91-135). Both of these deletion mutants were active, yielding hyperdorsalized embryos when injected as RNA. In addition, preliminary experiments demonstrated that noggin had an affinity for heparin, while a modified form with the basic region deleted did not possess this binding activity.

15 Native human noggin (hNG) exhibits low bioavailability in animal sera, likely due to its binding to extracellular matrix. Fc-tagged human noggin (hNG-Fc) has been shown to bind to BMP4 with very high affinity. (Zimmerman, L.B., et al., Cell 86: 599-606 (1996)).  
20 Modification of hNG has resulted in the identification of compounds which show improved bioavailability while retaining the ability to bind and antagonize Bone Morphogenetic Proteins (BMPs). The specific modifications resulting in altered biological properties involve deletion of amino acids identified as responsible for  
25 conferring heparin binding activity to the native noggin molecule. The resulting modified noggin lacks heparin binding activity but, unexpectedly, retains the ability to bind and antagonize BMP4.

Specifically, applicants have created two molecules, known as hNG $\Delta$ 138-144Fc and hNG $\Delta$ 133-144Fc. These molecules are Fc-tagged deletion muteins of human noggin lacking either amino acids 138 to 144 (for hNG $\Delta$ 138-144Fc) or 133 to 144 (for hNG $\Delta$ 133-144Fc) and tagged with the Fc domain of human IgG1 at their C-termini. To construct Fc-tagged noggins, a BspEI-NotI human IgG1 fragment (See Davis, S., et al., Science 266: 816-819 (1994); Economides, A.N., et al., Science 270: 1351-1353 (1995)) may be fused to human noggin using an oligonucleotide encoding a peptide bridge sequence as described by Zimmerman, L.B., et al., Cell 86: 599-606 (1996). As described below, the deletion muteins hNG $\Delta$ 138-144Fc and hNG $\Delta$ 133-144Fc displayed identical activity with hNG-Fc for binding to BMP4, as well as reduced affinity for heparin and superior pharmacokinetics in animal sera as compared to hNG.

#### Construction of hNG $\Delta$ 133-144Fc

hNG $\Delta$ 133-144Fc consists of the sequence of human noggin (hNG) with a deletion of amino acids 133 to 144 (numbering begins with the initiating methionine being amino acid number 1), fused to the constant region of human immunoglobulin G1 (Fc) via a Ser-Gly "bridge" (encoded by a genetically engineered *Bsp* EI restriction enzyme site) (Figure 14) (SEQ ID NO. 23). hNG $\Delta$ 133-144Fc was constructed by PCR and was cloned into the mammalian expression vector pMT21 (pMT21.hNG $\Delta$ 133-144Fc) using standard genetic engineering techniques. The correctness of the sequence of

hNG $\Delta$ 133-144Fc was verified by sequencing. Subsequently, this deletion mutein was also transferred to the expression vector pSRa, and both the Fc-tagged and the untagged version of this hNG mutein were constructed (pSRa.hNG $\Delta$ 133-144Fc, and  
5 pSRa.hNG $\Delta$ 133-144 respectively).

#### Expression of hNG $\Delta$ 133-144Fc

hNG $\Delta$ 133-144Fc was initially expressed in COS7 cells using a Lipofectamine (GIBCO/BRL) based transfection protocol. hNG $\Delta$ 133-  
10 144Fc, which like hNG is secreted, was purified from the conditioned media by passing through a Protein A-Sepharose column (Pharmacia) and eluting it with 100 mM acetic acid which was subsequently neutralized with Tris buffer to pH 7. The purity of this preparation was checked by SDS-PAGE followed by staining  
15 with the fluorescent dye SYPRO Orange (Molecular Probes, Inc.). The resulting preparation was more than 90% pure by this criteria and it was primarily in a disulfide-linked dimeric form, in agreement with previous observations made both with untagged hNG as well as with hNG-Fc, both of which also form disulfide-  
20 linked dimers.

#### Activity of hNG $\Delta$ 133-144Fc

##### Binding to BMP4:

To determine if this hNG mutein was active, its ability to bind to  
25 hBMP4 was compared with that of hNG-Fc, which binds human Bone Morphogenetic Protein 4 (hBMP4) with affinity essentially identical to that of untagged hNG. An ELISA format assay where

hBMP4 is captured on the surface of an ELISA plate was used to compare the two proteins. The hNG $\Delta$ 133-144Fc mutein displayed the same binding to BMP4 as hNG-Fc (Figure 15), indicating that the two proteins have the same biological activity.

5

#### Binding to Heparin:

We examined the heparin binding profile of hNG-Fc and compared it with that of hNG $\Delta$ 133-144Fc and another hNG deletion mutein which bears a shorter deletion in the basic region, namely, hNG $\Delta$ 138-144Fc (also referred to as hNG $\Delta$ KKLRRK-Fc). As shown on Figure 16, hNG-Fc starts to elute from heparin at about 0.75 M NaCl, whereas hNG $\Delta$ 133-144Fc and hNG $\Delta$ 138-144Fc start eluting at 0.25 M NaCl. Binding to heparin at above 0.5 M NaCl is considered to be an affinity interaction with heparin whereas binding to heparin below 0.5 M NaCl is considered to be due to ionic interactions (heparin contains sulfate groups which are negatively charged and hNG has regions which are positively charged). Therefore, it appears that the two hNG deletion muteins, hNG $\Delta$ 133-144Fc and hNG $\Delta$ 138-144Fc interact with heparin through ionic interactions, whereas hNG-Fc displays an affinity for heparin. Since the KKLRRK deletion (i.e. that embodied by hNG $\Delta$ 138-144Fc) is adequate for reducing the interaction with heparin to that expected for ionic effects, we conclude that the sequence KKLRRK (i.e.. amino acids 138 to 144 in hNG) is or contains the heparin binding domain of hNG. The Fc-domain did not bind to heparin.

25

We have also found that hNG and hNG-Fc display identical binding to

heparin by the method described above, whereas a chemically modified form of hNG (citraconyl-hNG) does not bind to heparin. We have also examined the heparin binding profile of another hNG deletion mutein that bears a longer deletion beyond the basic region, namely hNG $\Delta$ 133-179Fc. hNG $\Delta$ 133-179Fc displays the same binding profile to heparin as hNG $\Delta$ 133-144Fc and hNG $\Delta$ 138-144Fc, indicating that further deletion of the sequence downstream of the basic region does not further reduce the ionic component of hNG binding to heparin. From this we conclude that the ionic component of hNG binding to heparin must reside within the Kunitz-like domain of hNG. Furthermore, the chemically modified citraconyl-hNG and the two deletion muteins hNG $\Delta$ 138-144Fc and hNG $\Delta$ 133-144Fc are all biologically active (i.e., they bind to BMP4 with the same apparent affinity as hNG-Fc), whereas the deletion mutein hNG $\Delta$ 133-179Fc is inactive. Taken together, these results indicate that binding of hNG to heparin can be separated from binding to BMP4, and that the heparin binding domain of hNG is not required for its BMP4 blocking activity.

#### 20 Pharmacokinetic profile of hNG $\Delta$ 133-144Fc

The pharmacokinetic properties of hNG $\Delta$ 133-144Fc were tested in both mice and rats. It had been previously determined that unmodified hNG expressed in E. coli and refolded displays very poor bioavailability in rat serum after intravenous administration, with an apparent half life of less than 60 minutes. Citraconyl-hNG, which does not bind to heparin, displays a 30-fold improvement in



bioavailability but also disappears from circulation at about 2 hours post-injection. We reasoned that hNG $\Delta$ 133-144Fc which does not bind to heparin and which also has an Fc-tag may have a longer half-life in vivo. To examine this possibility, we injected mice and rats with hNG $\Delta$ 133-144Fc and determined its bioavailability in sera.

When hNG $\Delta$ 133-144Fc was injected into mice intraperitoneously (ip), it was detectable even in the latest time point sampled, which was 24 hours, achieving levels of 2  $\mu$ g/ml (Figure 17A). Intravenous injection in rat also showed favorable pharmacokinetics, although the latest serum sample was taken at 6 hours. In that experiment approximately 18  $\mu$ g/ml of hNG $\Delta$ 133-144Fc were detected (Figure 17B). Similar results have been achieved with hNG $\Delta$ 138-144Fc, indicating that deletion of the heparin binding domain of hNG is responsible for the improved pharmacokinetic profile of these molecules over hNG. Since the assay used to detect hNG $\Delta$ 133-144Fc post-ip or post-iv injection in animal sera relies on the ability of hNG $\Delta$ 133-144Fc to bind to BMP4, we also know that the hNG $\Delta$ 133-144Fc that is detected is functional and capable of interacting with BMP4. Furthermore, since hNG displays a very high affinity for BMP4, we anticipate that the levels of hNG $\Delta$ 133-144Fc achieved in these experiments will block BMP4 activity in animal model and in clinical situations.

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EXAMPLE 10 - Construction of a hNG $\Delta$ B2 mammalian  
expression plasmid pSR $\alpha$ .hNG $\Delta$ B2

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5 A mammalian expression vector containing the cDNA for the human  
noggin mutein hNG $\Delta$ 133-144 (without Fc tag) was constructed as  
follows. Henceforth, hNG $\Delta$ 133-144 may also be referred to as  
hNG $\Delta$ B2. Similarly, hNG $\Delta$ 138-144 may be referred to as hNG $\Delta$ B1.

10 The plasmid designated pCAE294, which contains the wild type  
human noggin cDNA (hNG) subcloned into the mammalian expression  
vector pSR $\alpha$ , was digested with the restriction enzymes EcoR I and  
Mlu I (New England Biolabs, Beverly, Massachusetts). This  
restriction digest released a 4kb DNA fragment that included the  
entire pSR $\alpha$  vector sequence as well as the C-terminal one-third  
15 (223 nucleotides) of wild type human noggin (hNG). The fragment  
was gel-purified by standard molecular biology techniques (See,  
for example, Sambrook, et al., Molecular Cloning, A Laboratory  
Manual, Cold Spring Harbor Laboratory; Current Protocols in  
Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-  
20 Interscience, NY).

The plasmid designated pCAE302, which contains the cDNA for  
hNG $\Delta$ 133-144-Fc (see Example 9) subcloned into the expression  
vector pMT21 (Genetics Institute, Inc.), was digested with the  
25 restriction enzymes EcoR I and Mlu I, which released a 500bp DNA  
fragment encoding the N-terminal two-thirds (439 nucleotides) of  
human noggin mutein that has a deletion of the sequence encoding

amino acids 133 to 144. This DNA fragment was also gel-purified. The 500bp DNA fragment and the 4kb DNA fragment were ligated according to standard techniques (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) to create the new mammalian expression vector pSR $\alpha$ .hNG $\Delta$ B2.

**EXAMPLE 11 -** Construction of a hNG $\Delta$ B2 prokaryotic expression plasmid pRG625

A prokaryotic expression vector containing the cDNA for the human noggin mutein hNG $\Delta$ 133-144 (hNG $\Delta$ B2) was constructed as follows.

15 A DNA fragment encoding the cDNA for human noggin (hNG) was  
PCR-amplified from a plasmid designated pUC-hNog#7 that  
contains the cDNA for human noggin subcloned into the vector pUC.  
The PCR reaction was performed using standard techniques (See  
Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold  
20 Spring Harbor Laboratory; Current Protocols in Molecular Biology,  
Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY)  
using the amplification primers:

25 EVD-84 (5'-TGTTTTTGCATGCAGCACTACCTGCACATCCGTCCGGCA-3')  
(SEQ. ID. NO. 24)

EVD-85 (5'-  
CGAAAACGGCCGTTATCAACAAGAACATTTACATTCAGAGATGA  
TCGGGTACTGG-3'

5 (SEQ. ID. NO. 25)

The resulting PCR-amplified 1005bp DNA fragment was digested with the restriction enzyme Sph 1 (New England Biolabs, Beverly, Massachusetts) and ligated into pRG09, a low-copy-number plasmid that contains the  $\beta$ -lactamase gene and the lacUV5 promoter, and which had been prepared for ligation by digestion with the restriction enzymes Sph I and Nru I. Following ligation and bacterial transformation, a clone containing the human noggin gene under the transcriptional control of the lacUV5 promoter was identified and named pRG292. The pRG292 plasmid was digested with the restriction enzymes Swa I and Bsm I to release a 1054bp DNA fragment. This DNA fragment was gel-purified by standard techniques and ligated into pCP146, a high-copy-number plasmid encoding the *kan1* gene, which had been prepared for ligation by digestion with the restriction enzymes Swa 1 and Bsm 1. The resulting plasmid was designated pRG301. In order to construct the  $\Delta$ B2 deletion (deletion of the sequence encoding amino acids 133-144), pRG301 was digested with the restriction enzymes Mlu I and Msc I to release a 238bp fragment internal to the human noggin gene (NT 378-615 of SEQ. ID. No. 1). This fragment was then replaced with a 202bp fragment internal to the hNG $\Delta$ B2 gene in pCAE302 (see Example 9 - pMT21.hNG $\Delta$ 133-144 Fc) which was

released by digestion with Mlu I and Msc I. The resulting prokaryotic expression plasmid was designated pRG625.

EXAMPLE 12 - Expression of hNG $\Delta$ B2 in *E. coli*

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5

The *E. coli* host strain RFJ141 containing the hNG $\Delta$ B2 expression plasmid pRG625 was grown in 40L of medium in a 60L bioreactor (ABEC, Inc., Allentown, PA). The minimal glucose medium contained Dibasic Potassium Phosphate (5 g/L), Monobasic Potassium  
10 Phosphate (1 g/L), Ammonium Sulfate (0.35 g/L), Sodium Citrate (1 g/L), Sodium Sulfate (1.3 g/L), Dow P2000 Antifoam (1 mL/L), Calcium Chloride (0.075 g/L), 30 g/L Glucose, and 3 mL/L of a trace element solution (3 mL/L) containing appropriate trace levels of iron sulfate, zinc sulfate, copper sulfate, manganese sulfate,  
15 cobalt chloride and sodium molybdate.

The fermentation was carried out at a temperature of 37<sup>o</sup> C. The pH of the fermentation was controlled at 7.0 using ammonia gas, and the dissolved oxygen at 20% by successively ramping pressure,  
20 agitation, and oxygen flow to maintain the setpoint. When an optical density of 50 was reached, induction of protein synthesis was initiated by addition of the chemical inducer isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). The fermentation was allowed to proceed for an additional 3 hours to allow for sufficient quantity  
25 of hNG $\Delta$ B2 protein to accumulate in the cells. The cells were harvested by tangential-flow filtration using Koch 500 NMWCO hollow fiber filters and diafiltered into a lysis buffer containing

20 mM Tris, 20mM EDTA, and 120 mM NaCl and frozen at -20 C.

#### EXAMPLE 13 - Refolding and Purification of hNG $\Delta$ B2

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5 Cells from the fermentation were thawed, resuspended in 20mM Tris, 20 mM EDTA, 1% Tween 80 buffer and disrupted using a Manton Gaulin Homogenizer at 8000 psig. Inclusion bodies were washed to remove contaminating *E. coli* proteins using a tangential-flow filtration system with Microgon Hollow fiber  
10 membranes (Microgon, CA) with a nominal pore size of 0.2 microns. The buffer used to wash the inclusion bodies contained 20 mM Tris, 20 mM EDTA and 1% Tween 80. The washed inclusion bodies were solubilized in 6M guanidine hydrochloride, 10 mM DTT. The protein was partially purified by passing the protein through a 100K  
15 ultrafiltration membrane (Millipore, MA) to remove high molecular weight contaminants and lipids. The protein was concentrated with a 10K filter (Millipore, MA) then diluted for refolding to a final concentration of 0.1 mg/mL in a buffer containing 1.5M guanidine hydrochloride, 20% glycerol, 20mM Tris, 2 mM EDTA,  
20 0.5mM oxydized glutathione, 5mM reduced glutathione. The protein was allowed to refold for 8 days at 4 C. The refolded protein was concentrated and diafiltered into a buffer containing 4M urea, 20mM sodium acetate, pH 4.0 and loaded onto 10 cm diameter column containing 2.8L SP Sepharose FF Resin (Pharmacia) which  
25 was equilibrated with 20 mM sodium acetate, 20% glycerol, 4M urea, 0.02% CHAPS. The column was washed with three column

volumes of the same buffer containing 350 mM NaCl. The protein was eluted with an increasing salt gradient (400 - 750 mM NaCl) over ten column volumes using the same buffer. An additional quantity of protein was eluted at pH 8.5 using 20mM Bicine, 20mM acetate, 20% glycerol, 4M urea, 500mM NaCl. The eluted protein was diluted four-fold to maintain solubility. The protein was concentrated and diafiltered into a buffer containing 4M urea, 20mM Bicine, pH 8.5 and loaded onto a 10 cm diameter column containing 2.8L Q Sepharose FF Resin (Pharmacia) equilibrated with Buffer A (4M urea, 20% glycerol, 50mM Bicine, 0.02% CHAPS, pH 8.5). The column was washed with Buffer A and eluted with a linear salt gradient from 100 mM to 300 mM NaCl over 7 column volumes at 100 mL/min. The fractions were analyzed by SDS PAGE and pooled to provide 1.8 grams of purified hNG $\Delta$ B2 protein.

#### EXAMPLE 14 - Pegylation of hNG $\Delta$ B2

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The deletion mutant hNG $\Delta$ B2 was modified by covalent attachment of Polyethylene glycol using the following procedure. Methoxy-PEG-propionaldehyde (MW=20,000) from Shearwater Polymers (Cat. No. M-ALD-20000) was reacted with 300 mg hNG $\Delta$ B2 (MW= 21,495) at a stoichiometric ratio of 20:1 PEG reagent to protein. The reaction mixture was filter sterilized and the reaction allowed to proceed at room temperature for 12 days under an argon atmosphere in a buffer containing 100 mM Bicine, 5 mM sodium cyanoborohydride, 20% glycerol, 4M purified urea, pH 8.1. The concentration of hNG $\Delta$ B2 in the reaction mixture was 0.75 mg/mL.

The urea was prepurified before use by passing it over a cation exchange column to remove contaminating amines. The use of the cyanoborohydride is necessary to reduce the Schiff base intermediate to a secondary amine linkage between the methoxy capped PEG and the reacted amino group of the protein. The  
5 reaction product contained a mixture of unPEGylated, monoPEGylated and polyPEGylated forms.

#### EXAMPLE 15 - Purification of PEG-hNG $\Delta$ B2

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10 Purification of monoPEGylated hNG $\Delta$ B2 was carried out using a two step chromatographic procedure involving cation exchange followed by size exclusion. In the first step, the reaction mixture was diluted by a factor of 5 with Buffer A containing 4M Urea, 15 mM  
15 sodium citrate, 20% glycerol, pH 5.5 and loaded onto a 5 cm diameter x 13 cm long chromatography column (Pharmacia XK50) packed with SP Sepharose HP cation exchange resin (Pharmacia) using a linear velocity of 45 cm/HR. After loading the column was washed with Buffer A and PEGylated hNG $\Delta$ B2 eluted using a linear gradient from  
20 0 to 500 mM NaCl in Buffer A over 6 column volumes using a linear velocity of 45 cm/HR. SDS PAGE gel analysis was carried out on the column fractions and those containing monoPEGylated hNG $\Delta$ B2 were pooled. The monoPEGylated protein (PEG-hNG $\Delta$ B2) was concentrated to 0.76 mg/mL using an 7 cm diameter Amicon stirred  
25 cell concentrator. The protein was loaded onto a 100 cm XK26 column (Pharmacia) containing 95 cm of Superdex 200 size exclusion resin (Pharmacia) which was equilibrated with 2M urea, 150 mM NaCl, 20% glycerol, 10mM sodium citrate, pH 5.5. The



protein was eluted at a flow rate of 3 mL/min. The fractions were analyzed by SDS PAGE and the appropriate fractions pooled. The pooled monoPEGylated hNG $\Delta$ B2 was dialyzed into 50mM Tris, 150 mM NaCl, 10% v/v glycerol, pH 7.5. The protein was aliquoted and  
5 stored frozen at -40 C.

EXAMPLE 16 - Comparison of the heparin binding  
profiles of hNG, PEG-hNG, Citraconyl-hNG,  
hNG $\Delta$ B1, hNG $\Delta$ B2, PEG-hNG $\Delta$ B2, and  
10 hNG $\Delta$ B2-Fc

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Proteins that display low affinity to heparin can be eluted from an affinity column earlier - i.e. at a lower NaCl concentration - than proteins that display a high affinity to heparin (Lobb, RR, et al,  
15 Anal Biochem Apr 154: 1-14 (1986). Binding to heparin at salt concentration between 0.1 and 0.5 M NaCl is attributed to ionic interactions with the sulfate groups that are present on heparin, whereas binding above 0.5 M NaCl is attributed to bona-fide heparin binding sites. We compared the ability of hNG and hNG muteins as  
20 well as tagged or chemically modified forms of hNG and hNG muteins to bind to heparin using a high resolution heparin affinity column, with the idea that low binding to heparin is a desirable property for a protein-based pharmaceutical destined for systemic delivery. High resolution affinity chromatography was the method  
25 of choice since it provides accurate and highly reproducible results and can reveal even small differences in heparin binding.

The heparin-binding profiles of hNG, PEG-hNG, citraconyl-hNG, hNG $\Delta$ B1, hNG $\Delta$ B2, PEG-hNG $\Delta$ B2, and hNG $\Delta$ B2-Fc were compared by sequentially injecting them on a TSK-5W Heparin column (Toso Haas, Montgomeryville, PA) as follows: 40 $\mu$ g each of the hNG, PEG-hNG, hNG $\Delta$ B2-Fc and hNG $\Delta$ B2; 9 $\mu$ g citraconyl-hNG; and 15 $\mu$ g of PEG-hNG $\Delta$ B2, each in a volume of 50 $\mu$ l. After each sample was injected it was subjected to salt gradient from 150 mM to 2 M NaCl in 100 mM Tris pH 7.6, and the elution profile of the proteins was monitored by A<sub>280</sub>.

10

hNG was found to elute at 1.2 M NaCl (Figs. 18A-18C), in agreement with previous experiments in which a batch elution method had been used. Chemically modifying hNG with 20,000 MW Polyethelene Glycol (PEG) to form PEG-hNG resulted in a molecule that displayed only marginally less affinity for heparin, eluting at approximately 1 M NaCl (Fig. 18A). In contrast, citraconyl-hNG, which is hNG that has been chemically modified with citraconyl groups at the positions of accessible Lysines (LYS) to effectively change these positively charged residues (at physiological conditions) to negatively charged residues, displayed extremely low affinity for heparin, eluting at 0.15 M NaCl (Fig 18A). This confirms that the basic region of hNG (amino acids 133 to 144; numbering from the initiating Methionine as number 1) which contains 5 LYS, is the heparin binding site of hNG for heparin.

20  
25

The deletion mutein hNG $\Delta$ B1 (also known as hNG $\Delta$ KKLRRK or hNG $\Delta$ 139-144 supra) displayed reduced binding for heparin, eluting

at approximately 0.75 M NaCl (Fig. 18B). The deletion mutein hNG $\Delta$ B2, which lacks two additional LYS residues, elutes even earlier in the gradient, at approximately 0.6 M NaCl (Fig. 18B). Further chemical modification of this mutein by a single molecule  
5 of PEG 20,000 at a stoichiometry of one molecule of PEG per hNG $\Delta$ B2 dimer (PEG-hNG $\Delta$ B2) resulted in a further reduction in the binding to heparin, eluting at 0.45 M NaCl (Fig. 18B). This is consistent with the idea that PEG imparts enhanced solubility to proteins to which it is covalently attached and may also be  
10 attributed to masking of some of the ionic interactions that can exist between hNG $\Delta$ B2 and heparin. Interestingly, the same type of reduction in binding is achieved (Fig. 18C) by tagging hNG $\Delta$ B2 with the constant region of human IgG1 (Fc) to make the chimeric protein hNG $\Delta$ B2-Fc.

15

#### 20 DEPOSIT OF MICROORGANISMS

The following were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 under the terms of the Budapest Treaty:

25

			<u>ATCC No.</u>	<u>Date of</u>
<u>Deposit</u>				
5	phage	hnogλ-5	75311	9-23-92
	phage	hnogλ-7	75309	9-23-92
	phage	hnogλ-9	75310	9-23-92
	phage	hnogλ-10	75308	9-23-92
	hybridoma	RP57-16	CRL 11446	8-25-93

10 While the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate, and not to limit, the scope of the invention.

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Fig. 1A

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      10      20      30      40
      *      *      *      *
ATG GAG CGC TGC CCC AGC CTA GGG GTC ACC CTC TAC GCC CTG GTG
M E R C P S L G V T L Y A L V>

      50      60      70      80      90
      *      *      *      *      *
GTG GTC CTG GGG CTG CGG GCG ACA CCG GCC GGC GGC CAG CAC TAT
V V L G L R A T P A G G Q H Y>

      100      110      120      130
      *      *      *      *
CTC CAC ATC CGC CCG GCA CCC AGC GAC AAC CTG CCC CTG GTG GAC
L H I R P A P S D N L P L V D>

      140      150      160      170      180
      *      *      *      *      *
CTC ATC GAA CAC CCA GAC CCT ATC TTT GAC CCC AAG GAA AAG GAT
L I E H P D P I F D P K E K D>

      190      200      210      220
      *      *      *      *
CTG AAC GAG ACG CTG CTG CGC TCG CTG CTC GGG GGC CAC TAC GAC
L N E T L L R S L L G G H Y D>

      230      240      250      260      270
      *      *      *      *      *
CCA GGC TTC ATG GCC ACC TCG CCC CCC GAG GAC CGG CCC GGC GGC
P G F M A T S P P E D R P G G>

      280      290      300      310
      *      *      *      *
GGC GGC GGT GCA GCT GGG GGC GCG GAG GAC CTG GCG GAG CTG GAC
G G G A A G G A E D L A E L D>

      320      330      340      350      360
      *      *      *      *      *
CAG CTG CTG CGG CAG CGG CCG TCG GGG GCC ATG CCG AGC GAG ATC
Q L L R Q R P S G A M P S E I>

      370      380      390      400
      *      *      *      *
AAA GGG CTA GAG TTC TCC GAG GGC TTG GCC CAG GGC AAG AAG CAG
K G L E F S E G L A Q G K K Q>

      410      420      430      440      450
      *      *      *      *      *
CGC CTA AGC AAG AAG CTG CGG AGG AAG TTA CAG ATG TGG CTG TCG
R L S K K L R R K L Q M W L W>

      460      470      480      490
      *      *      *      *
TCG CAG ACA TTC TGC CCC GTG CTG TAC GCG TGG AAC GAC CTG GGC
S Q T F C P V L Y A W N D L G>

      500      510      520      530      540
      *      *      *      *      *
AGC CGC TTT TGG CCG CGC TAC GTG AAG GTG GGC AGC TGC TTC AGT
S R F W P R Y V K V G S C F S>

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Fig. 1B

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      550      560      570      580 —
      •       •       •       •
AAG CGC TCG TGC TCC GTG CCC GAG GGC ATG GTG TGC AAG CCG TCC
K   R   S   C   S   V   P   E   G   M   V   C   K   P   S>

      590      600      610      620      630
      •       •       •       •       •
AAG TCC GTG CAC CTC ACG GTG CTG CCG TGG CGC TGT CAG CCG CGC
K   S   V   H   L   T   V   L   R   W   R   C   Q   R   R>

      640      650      660      670
      •       •       •       •
GGG GGC CAG CGC TGC GGC TGG ATT CCC ATC CAG TAC CCC ATC ATT
G   G   Q   R   C   G   W   I   P   I   Q   Y   P   I   I>

      680      690
      •       •
TCC GAG TGC AAG TGC TGC TAG
S   E   C   K   C   S   C   *>

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Fig. 2A.

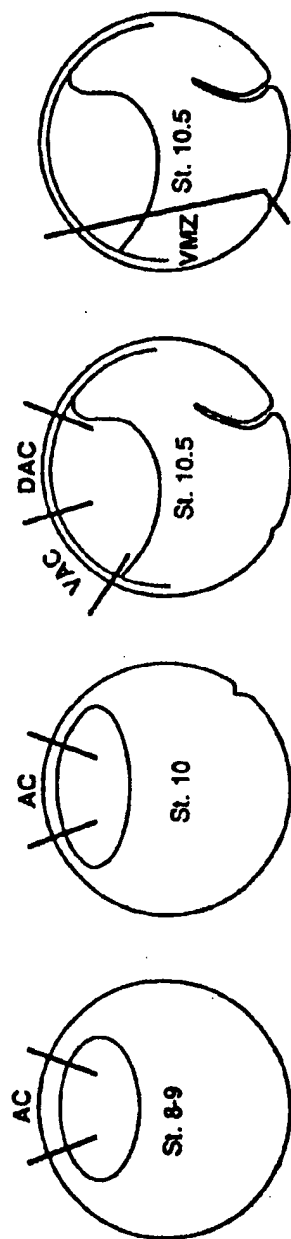
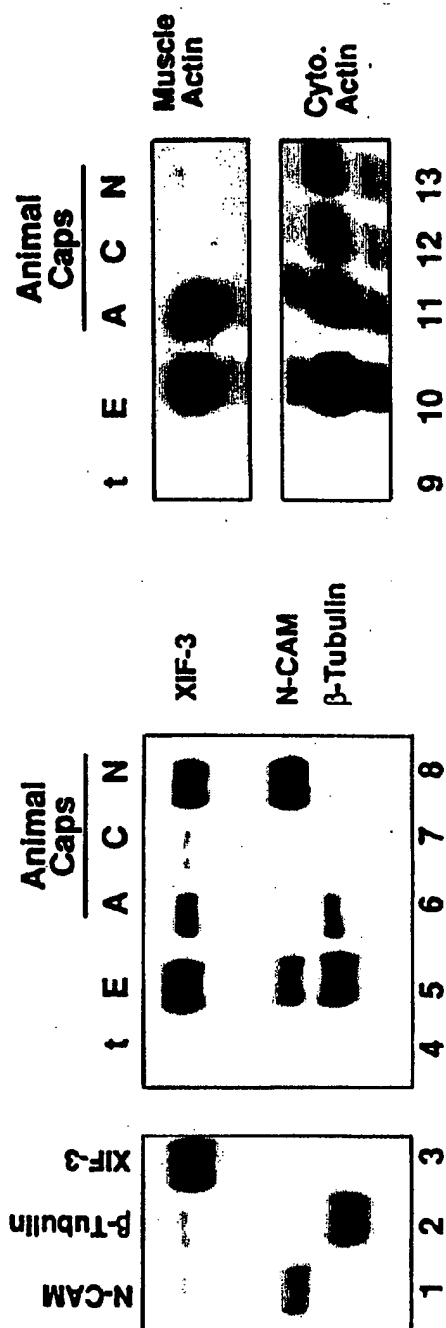
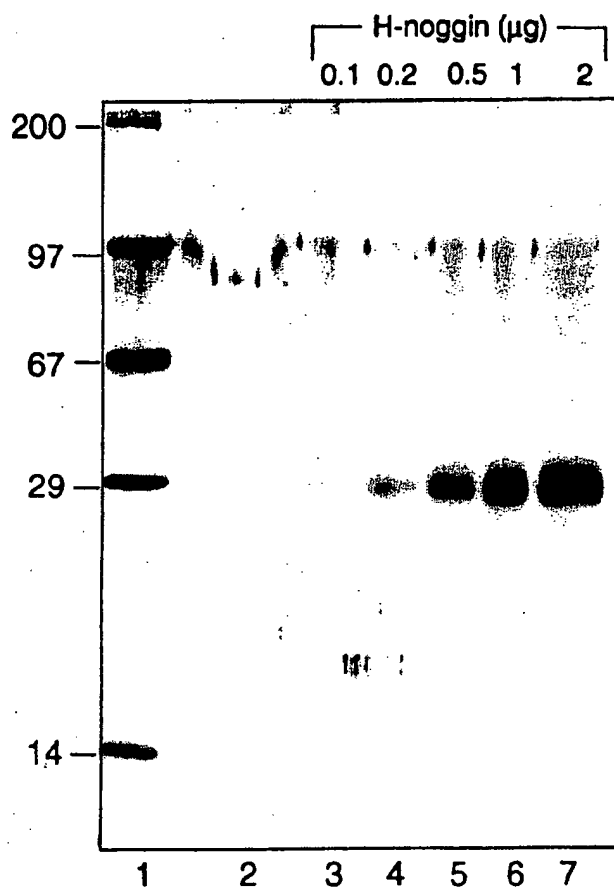


Fig. 2B.



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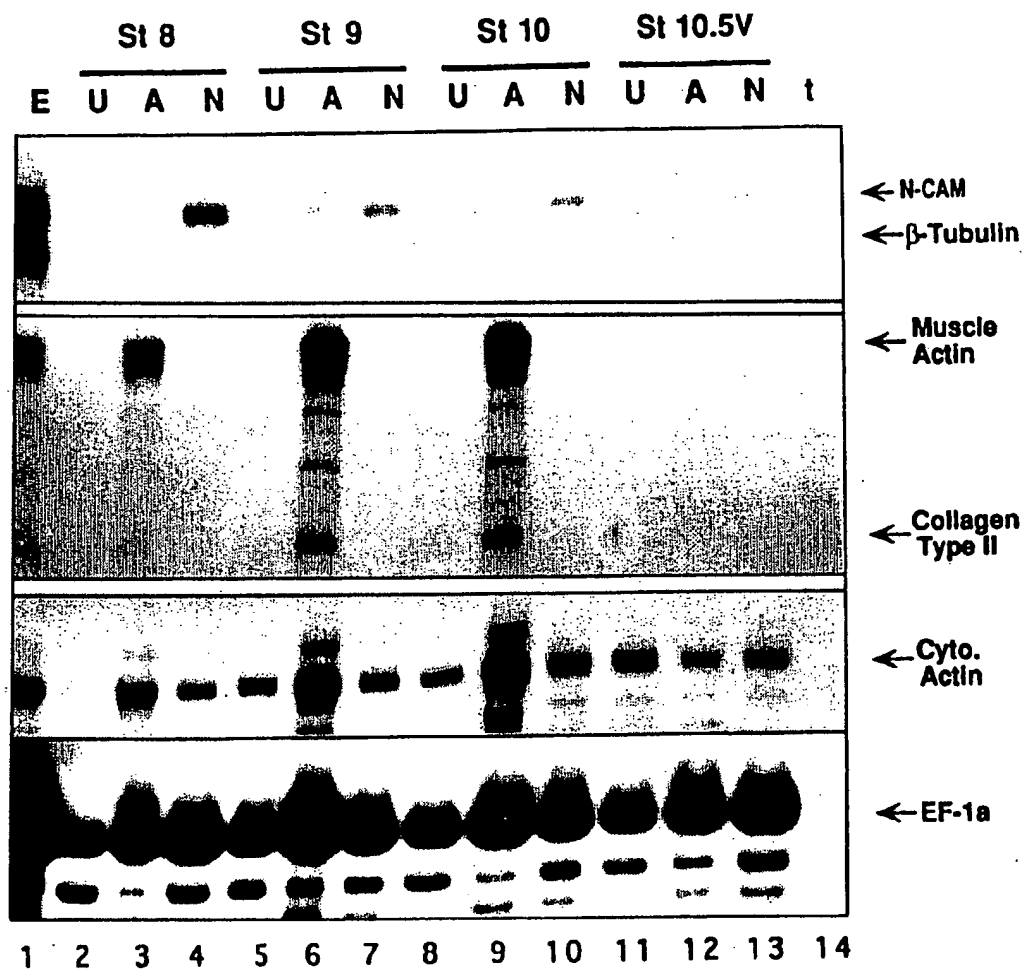
Fig. 3.

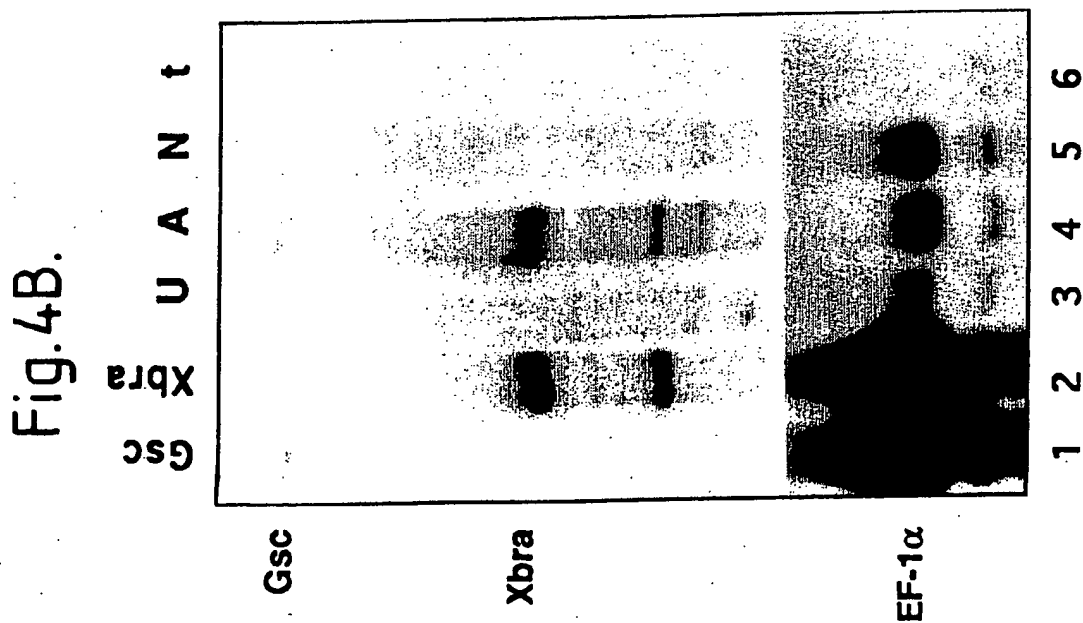
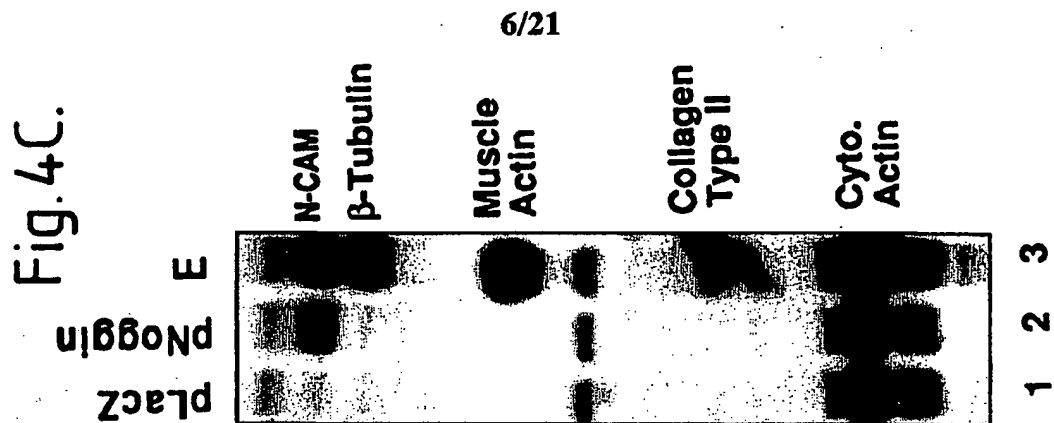




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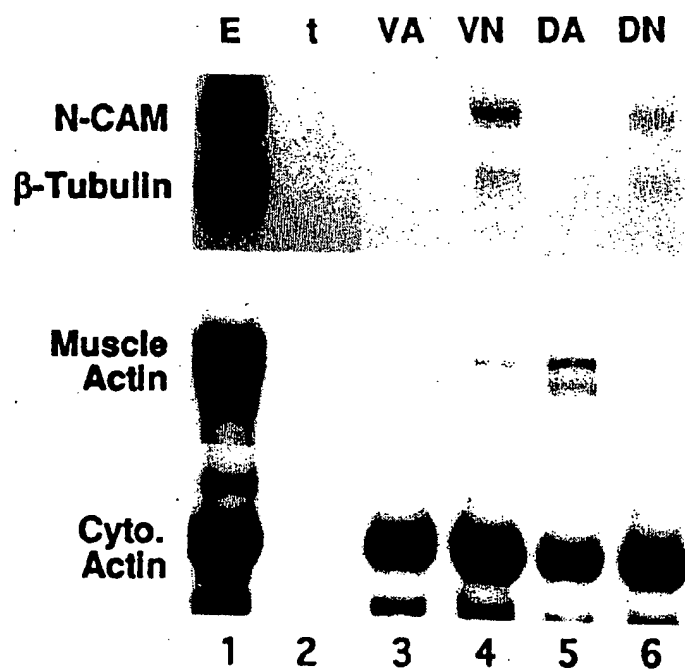
Fig. 4A.





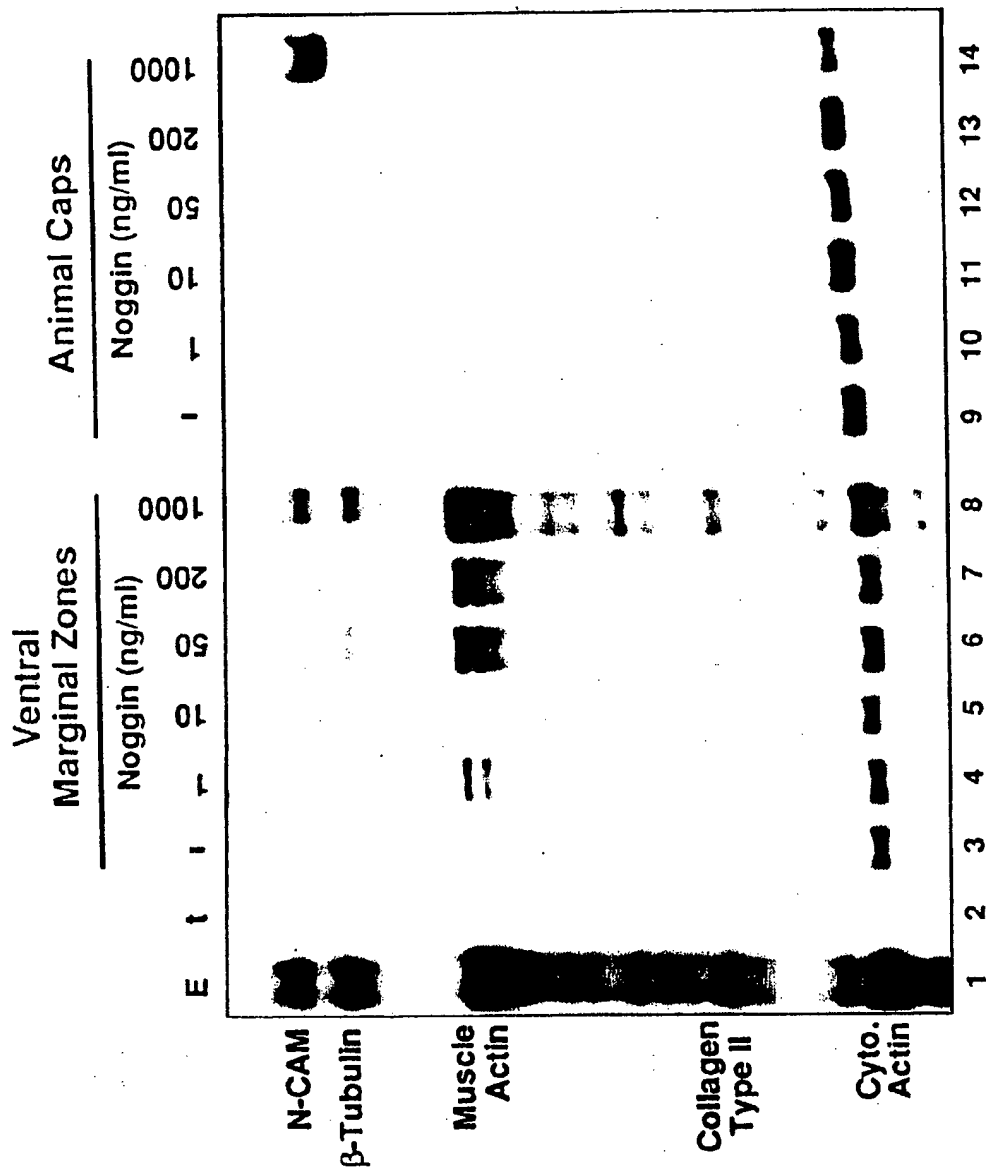
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Fig.5.



SUBSTITUTE SHEET (RULE 26)

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Fig. 7B.

N-CAM



Fig. 7A.

N-CAM



Fig. 7C.

Muscle Actin



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Fig. 7F.

Untreated

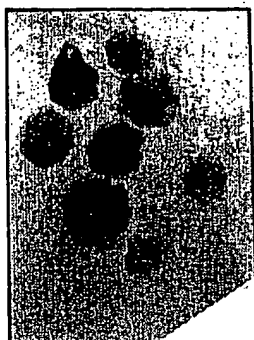


Fig. 7I.

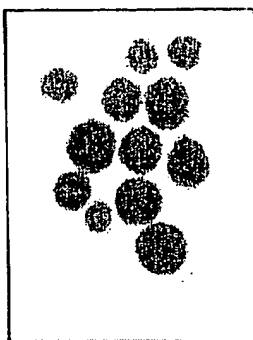


Fig. 7L.

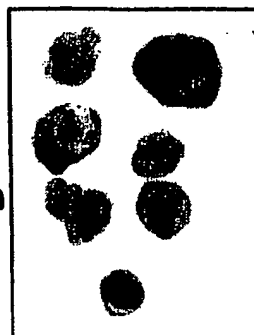


Fig. 7E.

+ Noggin

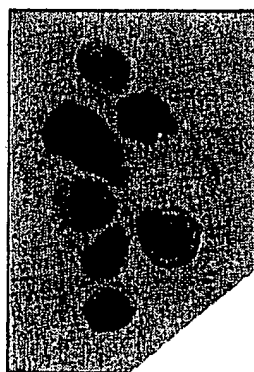


Fig. 7H.

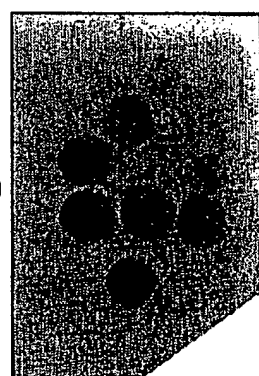


Fig. 7K.

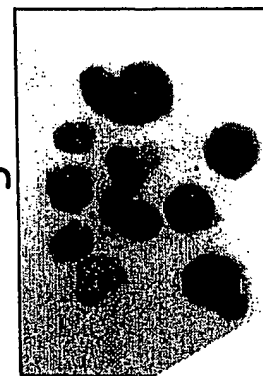


Fig. 7D.

Embryo

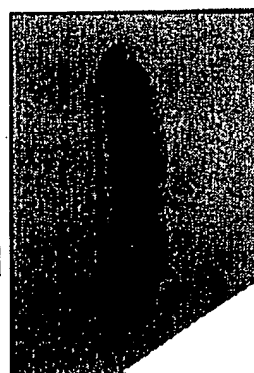
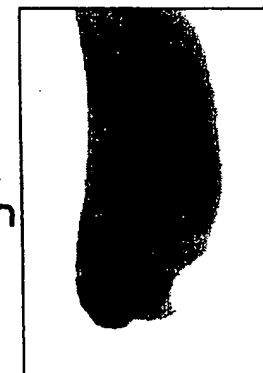


Fig. 7G.



Fig. 7J.



6F11

XAG

OTX

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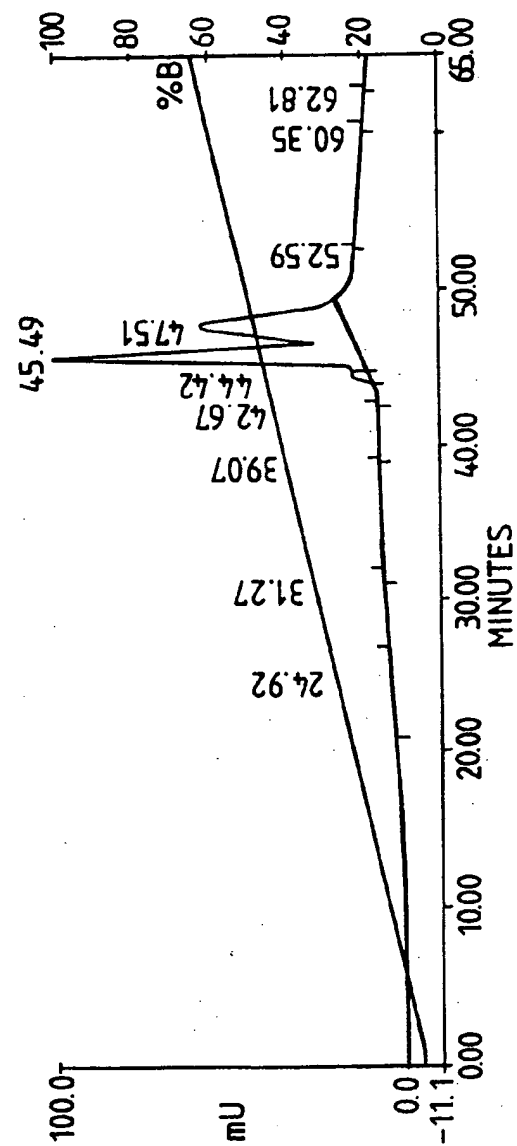


Fig. 8.

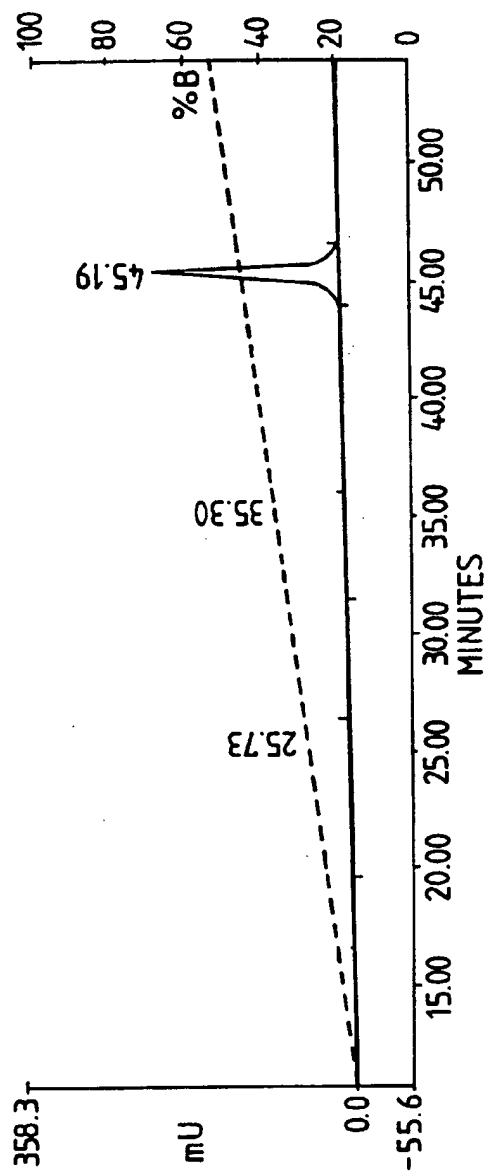


Fig. 9.

Fig.10

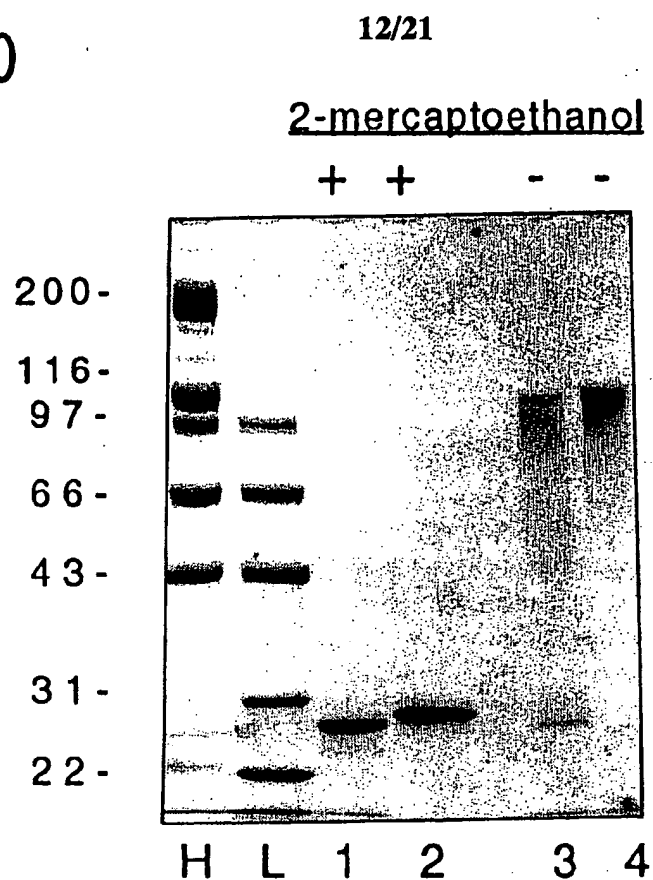
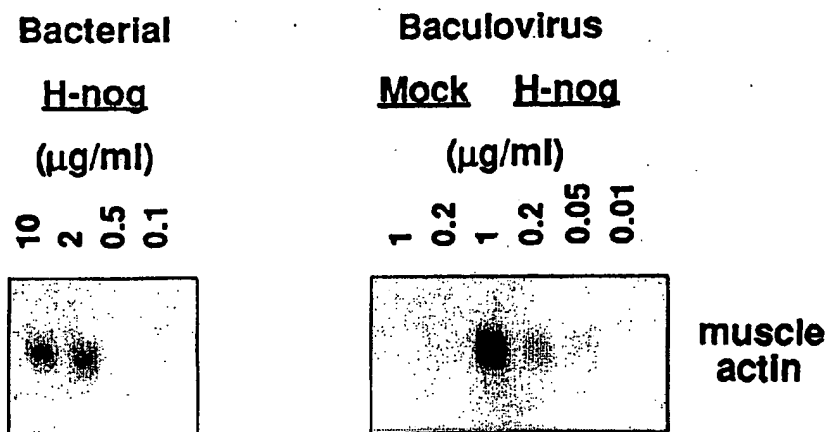


Fig.12.





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Fig. 11.

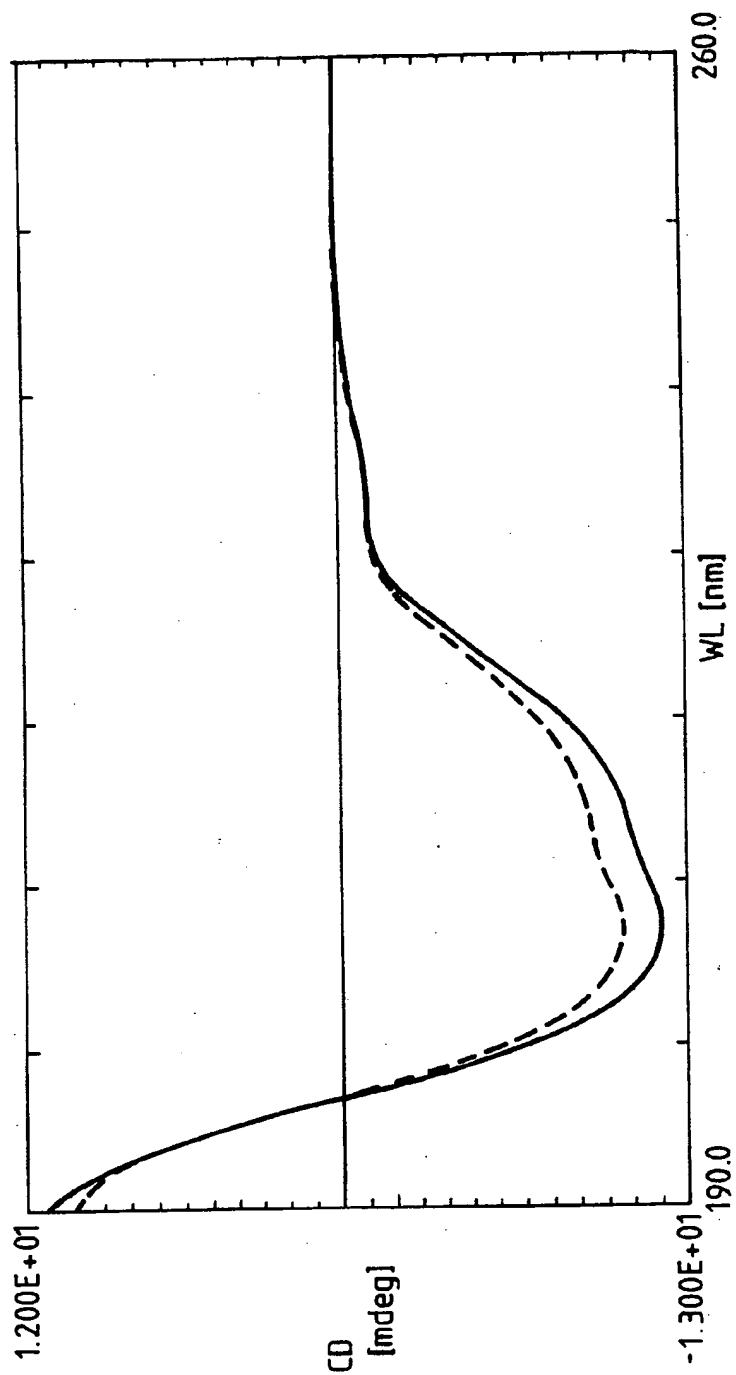


Fig. 13A

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```

      5   10   15   20   25   30   35   40   45   50   55   60
      *   *   *   *   *   *   *   *   *   *   *   *
TAACTCACTC ATTAGNCACC CCAGCCTTAC ACTTTATGCT TCCGGCTCGT ATGTTGTGTG

      65   70   75   80   85   90   95  100  105  110  115  120
      *   *   *   *   *   *   *   *   *   *   *   *
GAATTGTGAG CGGATAACAA TTTCACACAG GAAACAGCTA TGACCATGAT TACGCCAAGC

      125  130  135  140  145  150  155  160  165  170  175  180
      *   *   *   *   *   *   *   *   *   *   *   *
TCGAAATTAA CCCTCACTAA AGGGAACAAA AGCTGGAGCT CCACCGCGGT GGCGGCCGCC

      185  190  195  200  205  210  215  220  225  230  235  240
      *   *   *   *   *   *   *   *   *   *   *   *
TTCCCAAGTA GAGCGGCGGG GGGGAATTGC GACCAACTCG TGCGCGTCTT CTGCNCCGCG

      245  250  255  260  265  270  275  280  285  290  295  300
      *   *   *   *   *   *   *   *   *   *   *   *
GCGGGAGCCG GCGCTGCGCG AACGGCTCTC CTCGCAGCTC ATGCTGCCTG CCCTGCGCCT

      305  310  315  320  325  330  335  340  345  350  355  360
      *   *   *   *   *   *   *   *   *   *   *   *
GCTCAGCCTC GGGTGAGCCA CCTCCGGAGG GACCGGGGAG CGCGGCAGCG CCGCGGACTC

      365  370  375  380  385  390  395  400  405  410  415  420
      *   *   *   *   *   *   *   *   *   *   *   *
GGCGTGCTCT CCTCCGGGGA CGCGGGACGA AGAGGCAGCC CCGGGGCGCG CGCGGGAGGC

      425  430  435  440  445  450  455  460  465  470  475  480
      *   *   *   *   *   *   *   *   *   *   *   *
ATGGAGCGCT GCCCCAGCCT GGGGGTCACC CTCTACGCCC TGGTGGTGGT CCTGGGGCTG
M E R   C P S L   G V T   L Y A   L V V V   L G L >

      485  490  495  500  505  510  515  520  525  530  535  540
      *   *   *   *   *   *   *   *   *   *   *   *
CGGGCAGCAC CAGCCGGCGG CCAGCACTAT CTACACATCC GCCCAGCACC CAGCGACAAC
R A A   P A G G   Q H Y   L H I   R P A P   S D N>

      545  550  555  560  565  570  575  580  585  590  595  600
      *   *   *   *   *   *   *   *   *   *   *   *
CTGCCCTTGG TGGACCTCAT CGAACATCCA GACCCTATCT TTGACCCTAA GGAGAAGGAT
L P L   V D L I   E H P   D P I   F D P K   E K D>

      605  610  615  620  625  630  635  640  645  650  655  660
      *   *   *   *   *   *   *   *   *   *   *   *
CTGAACGAGA CGCTGCTGCG CTCGCTGCTC GGGGGCCACT ACGACCCGGG CTTTATGGCC
L N E   T L L R   S L L   G G H   Y D P G   F M A>

      665  670  675  680  685  690  695  700  705  710  715  720
      *   *   *   *   *   *   *   *   *   *   *   *
ACTTCGCCCC CAGAGGACCG ACCCGGAGGG GGCGGGGGAC CGGCTGGAGG TGCCGAGGAC
T S P   P E D R   P G G   G G G   P A G G   A E D>

      725  730  735  740  745  750  755  760  765  770  775  780
      *   *   *   *   *   *   *   *   *   *   *   *
CTGGCGGAGC TGGACCAGCT GCTGCGGCAG CGGCCGTCGG GGGCCATGCC GAGCGAGATC
L A E   L D Q L   L R Q   R P S   G A M P   S E I>

      785  790  795  800  805  810  815  820  825  830  835  840
      *   *   *   *   *   *   *   *   *   *   *   *

```

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Fig. 13B

```

AAAGGGCTGG AGTTCTCCGA GGGCTTGGCC CAAGGCAAGA AACAGCGCCT GAGCAAGAAG
K G L E F S E G L A Q G K K Q R L S K K>

845 850 855 860 865 870 875 880 885 890 895 900
      *      *      *      *      *
CTGAGGAGGA AGTTACAGAT GTGGCTGTGG TCACAGACCT TCTGCCCGGT GCTGTACGCG
L R R K L Q M W L W S Q T F C P V L Y A>

905 910 915 920 925 930 935 940 945 950 955 960
      *      *      *      *      *
TGGAATGACC TAGGCAGCCG CTTTGGCCA CGCTACCTGA AGGTGGGCAG CTGCTTCAGC
W N D L G S R F W P R Y V K V G S C F S>

965 970 975 980 985 990 995 1000 1005 1010 1015 1020
      *      *      *      *      *
AAGCGCTCCT GCTCTGTGCC CGAGGGCATG GTGTGTAAGC CATCCAAGTC TGTGCACCTC
K R S C S V P E G M V C K P S K S V H L>

1025 1030 1035 1040 1045 1050 1055 1060 1065 1070 1075 1080
      *      *      *      *      *
ACGGTGCTGC GGTGGCGCTG TCAGCGCGCG GGGGGTCAGC GCTGCGGCTG GATTCCCATC
T V L R W R C Q R R G G Q R C G W I P I>

1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140
      *      *      *      *      *
CAGTACCCCA TCATTCCGA GTGTAAGTGT TCCTGCTAGA ACTCGGGGGG GGGCCCTGCC
Q Y P I I S E C K C S C *>

1145 1150 1155 1160 1165 1170 1175 1180
      *      *      *      *
CGCGCCCA CACTTGATGG ATCCCCGGG CTGAGATTTT

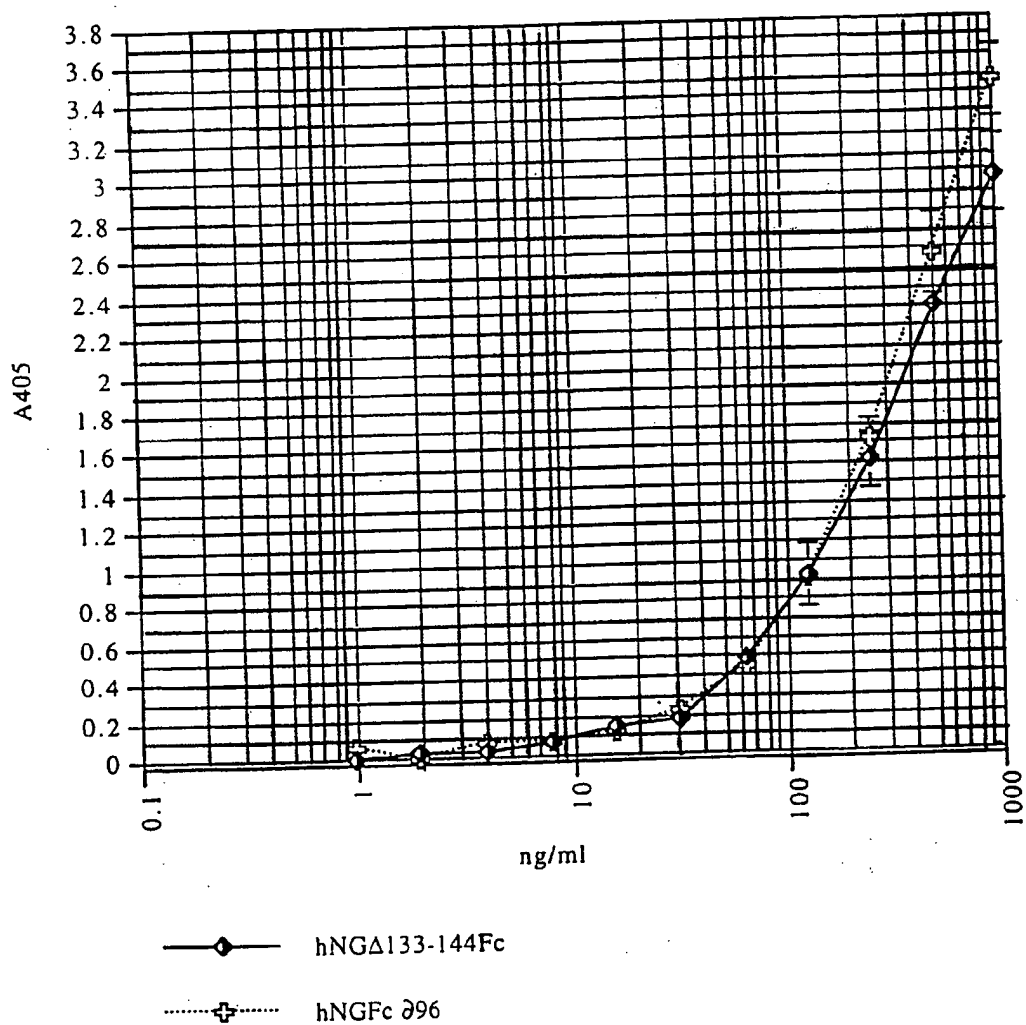
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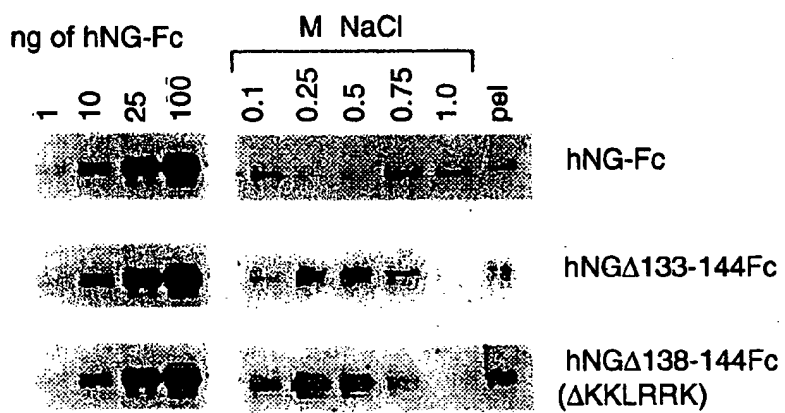
Fig. 14

	10	20	30	40	50	60	70	80
↑ *		*	*	*	*	*	*	*
MERCP	SLGVT	LYALVV	LGL	RATPAGG	QHYL	HIRPAPSDN	LPLVD	LIEHPDPIFD
								KEKD
								LNETLLRSL
								LLGGHYDP
								GFMA
90	100	110	120	130	140	150	160	
*	*	*	*	*	*	*	*	*
TSPPE	DRPGG	GGAAG	AED	LAELD	QLLRQ	RPSGAMP	SEI	KGLEF
								SEGLAQGLQ
								MWLSQ
								TFCPVLYA
								WNDLGS
								RFRWPRY
170	180	190	200	210	220	230	240	
↑ *	↑ *	↑ *	↑ *	↑ *	↑ *	↑ *	↑ *	↑ *
VKVG	SCFS	KRSC	SVPE	GMVC	KPSK	SVHL	TVLR	WRCOR
								RG
								QRCGW
								IPIQYPII
								SECKCSC
								SGDKTHTC
								PPCPAPE
								LLGGP
250	260	270	280	290	300	310	320	
*	*	*	*	*	*	*	*	*
SVFL	FPKPK	DTLM	ISRTPE	VTCA	VVDV	SHED	PEVK	ENWY
								VDGVEV
								HNNAKTK
								PREEOYNS
								TYRVVS
								VLTVL
								HODW
								LNKGKE
330	340	350	360	370	380	390	400	
↑ *	↑ *	↑ *	↑ *	↑ *	↑ *	↑ *	↑ *	↑ *
YKCV	SNKAL	PAP	IEKTISK	AKG	PREPO	VTVL	PPS	REEM
								TKNOV
								SLTCLV
								KGYF
								PSDIA
								VEWES
								NGOPEN
								NNYKTT
								PTPVL
410	420	430	440	450				
*	*	*	*	*	*	*	*	*
DSDE	SFFLY	SKLT	VDK	SRWO	OGNV	FESC	VMHE	ALHNHYTO
								KSLSLSPGK

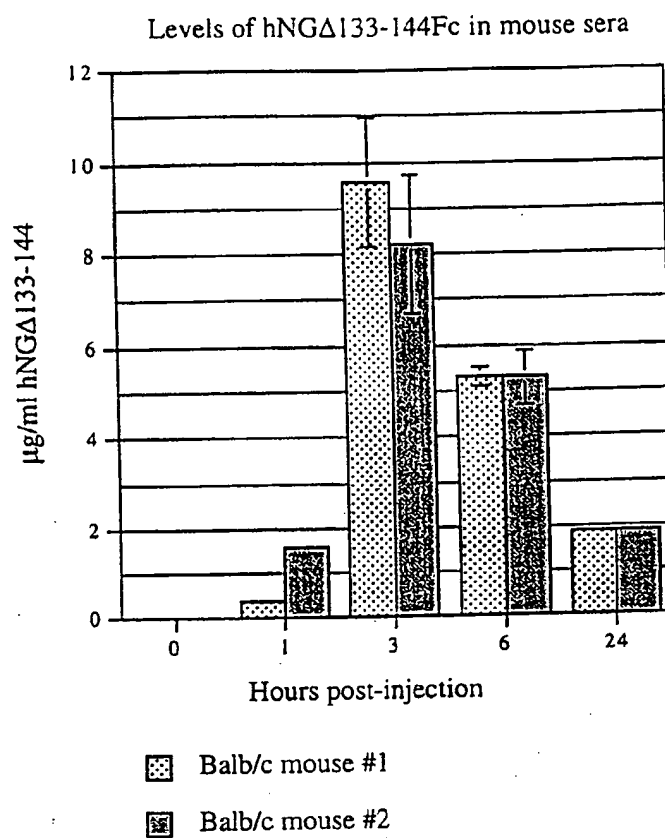
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**Fig. 15**

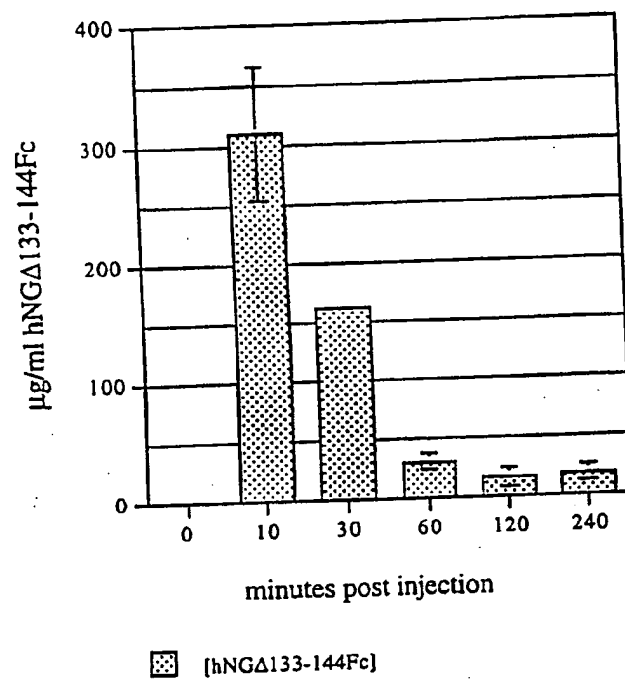
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**Fig. 16**

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**Fig. 17A**

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**Fig. 17B**Levels of hNG $\Delta$ 133-144Fc in rat sera



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Fig. 18A

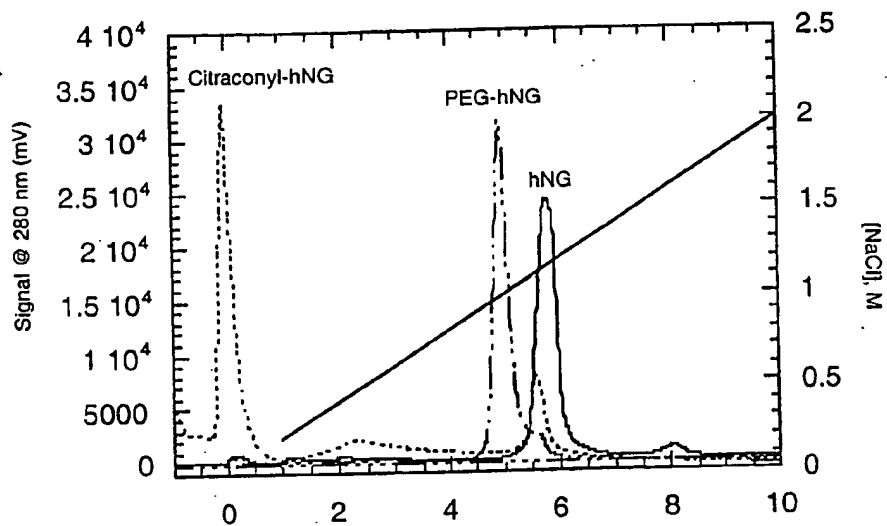


Fig. 18B

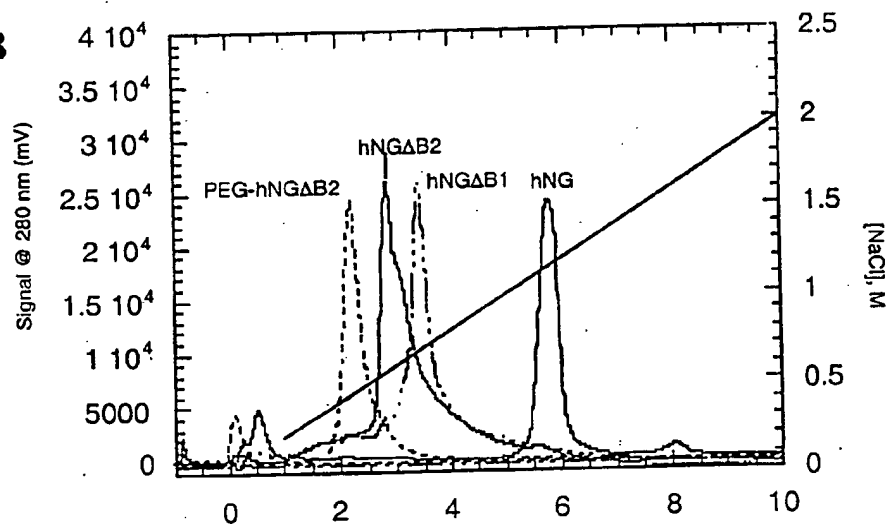
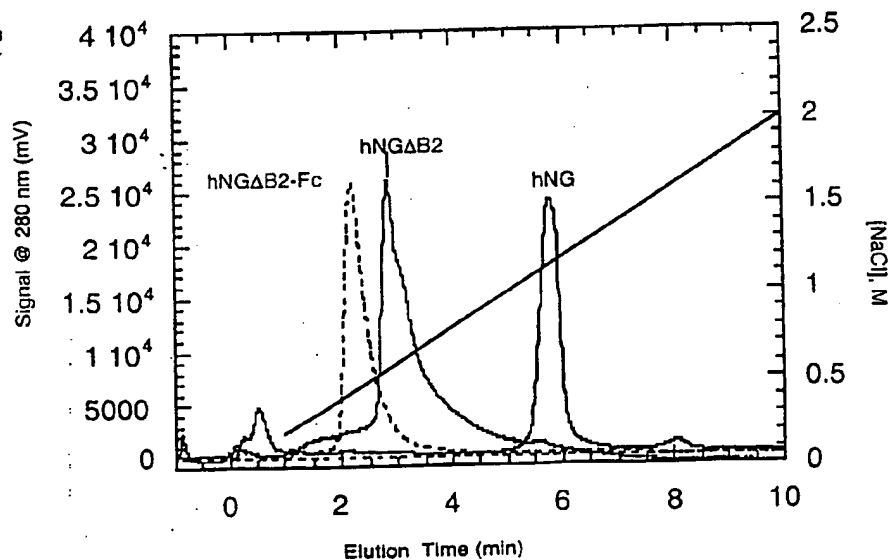


Fig. 18C



# INTERNATIONAL SEARCH REPORT

Inter. Appl. No.

PCT/US 98/14603

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/475 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZIMMERMAN L B ET AL: "THE SPEMANN ORGANIZER SIGNAL NOGGIN BINDS AND INACTIVATES BONE MORPHOGENETIC PROTEIN 4" CELL, vol. 86, 23 August 1996, pages 599-606, XP002066229 cited in the application see page 604, left-hand column	1-35
A	VALENZUELA D M ET AL: "IDENTIFICATION OF MAMMALIAN NOGGIN AND ITS EXPRESSION IN THE ADULT NERVOUS SYSTEM" THE JOURNAL OF NEUROSCIENCE, vol. 15, no. 9, September 1995, pages 6077-6084, XP002087688 see figure 1	1-35

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"8" document member of the same patent family

Date of the actual completion of the international search

14 December 1998

Date of mailing of the international search report

29/12/1998

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European Patent Office, P.B. 5618 Patentlaan 2  
NL - 2280 HV Rijswijk  
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Fax: (+31-70) 340-3016

Authorized officer

Cupido, M

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/14603

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SMITH W C ET AL : "EXPRESSION CLONING OF NOGGIN, A NEW DORSALIZING FACTOR LOCALIZED TO THE SPEMANN ORGANIZER IN XENOPUS EMBRYOS"</p> <p>CELL, vol. 70, 4 September 1992, pages 829-840, XP002087689 NA US cited in the application see figures 2A,2B</p> <p style="text-align: center;">---</p>	1-35
A	<p>LEMAIRE P ET AL: "THE VERTEBRATE ORGANIZER: STRUCTURE AND MOLECULES" TRENDS IN GENETICS., vol. 12, no. 12, December 1996, pages 525-531, XP002087690 AMSTERDAM NL see the whole document</p> <p style="text-align: center;">-----</p>	1-35

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/14603

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 9, 18, 27 and 30-33  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.